ORGANIZATION OF $GABA_A$ RECEPTOR SUBTYPES IN SPINAL NOCICEPTIVE PATHWAYS

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Jolly Paul

aus

Kerala, India

Promotionkomitee

Prof. Dr. Jean-Marc Fritschy, examiner

Prof. Dr. Hanns Ulrich Zeilhofer, co-examiner

Prof. Dr. Esther Stöckli, co-examiner

Zurich, 2012

Dedicated to

Mamma and Shank

Acknowledgements

Pursuing a PhD project was an incomparable experience in life that in fact shaped me as a person. It was like climbing a peak, step by step, accompanied with joy, bitterness, hardships, frustration, encouragement and trust and with so many people's kind help. I am truly privileged to thank all of them for their unequivocal roles in facilitating an efficient team work for these projects.

First of all, I would like to extend my heart-felt gratitude to my honorific supervisors, Prof. Dr. Hanns Ulrich Zeilhofer and Prof. Dr. Jean-Marc Fritschy for giving me the great opportunity to conduct this challenging research project at the Institute of Pharmacology and Toxicology, with its excellent infrastructure. I would like to express my sincere gratefulness to Prof. Dr. Hanns Ulrich Zeilhofer for his excellent co-supervision in the collaborative projects given to me. Thank you for giving me the responsibility of multiple projects, thus helping me to grow as an efficient and sharp minded person. I am extremely grateful for his foresights, intensive encouragement and constructive feedback for the accomplishment of my thesis.

Prof. Dr. Jean-Marc Fritschy has been the most encouraging supervisor and a great teacher guiding me patiently with advices, fruitful discussions and timely interventions. During these years, he passed on to me his fascination and enthusiasm for microscopy and morphology. I have learned a lot from his expertise and experience in neuroscience and without his help I could not have finished my dissertation successfully. Thank you Prof. Dr. Jean-Marc Fritschy and Prof. Dr. Hanns Ulrich Zeilhofer for creating an excellent collaborative project team with your unequivocal expertise from which I could tremendously benefit.

I would also like to thank Prof. Dr. Esther Stöckli for agreeing to be co-reviewer of this PhD thesis and my thesis committee member within the Zurich Neuroscience Center (ZNZ). I have greatly benefited from her advices and critical comments during these four years. I would also like to thank Prof. Dr. Stephen B. McMahon, Prof. Dr. Andrew Todd and Dr. Ping Yip for their inputs and guidance for the experiments.

Corinne Sidler, Dr. Robert Witschi and Dr. Caroline Petitjean introduced me to immunohistochemistry, behavioral experiments and confocal microscopy and I would like to extend my sincere thanks to all of them. I am gratefully indebted to Prof. Dr. Patrizia

Panzanelli, Dr. Dietmar Benke and Dr. Florence Crestani for their help with electron microscopy, biochemical, behavioral experiments and fruitful discussions. Many thanks to Isabelle Camenisch, Christina Köster, Thomas Grampp, Louis Scherrer and Ruth Keist without whom I would not have had such a smooth path towards the completion of my dissertation.

My sincere gratitude goes to Christel Genoud, Jana Döhner, Gery Barmettler and Therese Bruggmann for sharing your great knowledge in the amazing area of electron microscopy. Thank you for all the advice and professional support throughout my PhD. I am deeply grateful to Cornelia Leuzinger, Ana Hemmersbach and Edward Apau for their unlimited assistance in the animal house facility. My sincere thanks also go to Romy Gisler, Katrin Uster, Verena Duenki and Stefanie Rebstein for their administrative support during all these years.

Special thanks go to William Ralvenius, Sabine Haueter, Alessandra Di Lio, Dr. Gonzalo Yévenes and Dr. Pradeep Punnakkal for being excellent team mates and friends in various projects. I am blessed with excellent lab mates, office mates and desi mates and I would like to thank all of them for making my life much easier. A special and warm thanks to Dr. Helge Johannssen, Dr. Lena Matveeva, Dr. Marta Grabiec, Nandkishore Mule and Dr. Shanker Jha for their help in time at the various stages of this thesis preparation. I would like to thank all the other members of Fritschy and Zeilhofer groups and of the institute, for discussion and support, and for providing me with a nice working atmosphere.

Last but not least, I would like to thank my parents (especially Mamma and Pappa) for their excellent support throughout my whole life. Big thanks to my husband Bijoy for his support, encouragement and advices during these years. It is also a great pleasure to thank my sincere and dedicated friends Shank, Hari and Mabel for their inspiring and illimitable friendship.

Thank you all…….

Jolly Paul
Zurich, January 2012

Table of Contents

I Summary

Chronic pain is a frequent neurological condition occurring at least in part as the consequence of progressive changes in the nociceptive circuits of the peripheral and central nervous system. It frequently arises from tissue damage, inflammation or from damage of neurons and nerves. Diminished synaptic inhibition in the spinal dorsal horn is a major contributor to chronic pain syndromes. Classical benzodiazepines (BDZ) facilitate synaptic inhibition through a positive allosteric modulation of $GABA_A$ receptors ($GABA_AR$). They exert antihyperalgesic actions after spinal application in rodents and humans, but lack general analgesic activity in humans after systemic administration. Furthermore, in particular their long-term use is limited by unwanted drug effects such as sedation, memory impairment, tolerance development and addiction. $GABA_AR$ form multiple pharmacologically and functionally distinct subtypes, best differentiated by the type of α subunit. The spinal antihyperalgesic effect of BDZ in rodents is mediated mainly by α2 and α3 containing $GABA_AR$ (α2 and α3 $GABA_AR$), while most of the adverse effects are caused by α1 $GABA_AR$ located at supraspinal sites. Novel subtype-selective $GABA_AR$ ligands sparing α1 $GABA_AR$ might thus exert antihyperalgesia in the absence of major unwanted effects. To strengthen the scientific basis for such an approach, we aimed at a better understanding of the GABAergic circuits responsible for the antihyperalgesic effects of dorsal horn $GABA_AR$.

In the spinal dorsal horn, $GABA_AR$ are expressed not only postsynaptically on intrinsic neurons, but are also found at the spinal terminals of primary afferent nociceptors where they contribute to presynaptic inhibition through primary afferent depolarization (PAD). Many of these presynaptic $GABA_AR$ belong to the α2 $GABA_AR$. In the first project of this thesis, experiments were performed with nociceptor-specific α2 $GABA_AR$-deficient (*sns*-$\alpha2^{-/-}$) mice and conditional (*sns*-$\alpha2^{R/-}$) point-mutated mice, whose primary nociceptor α2 $GABA_AR$ subunits have been rendered diazepam-insensitive. The results revealed unchanged baseline nociceptive thresholds, and unchanged inflammatory and neuropathic hyperalgesia, but decreased antihyperalgesia evoked by intrathecally applied diazepam in an inflammatory pain model.

The second project aimed at the identification of a possible contribution of supraspinal α2 $GABA_AR$ to antihyperalgesia, e.g. through a reversal of anxiety-induced hyperalgesia or through a genuine antihyperalgesic effect. To this end, *Hoxb8*-$\alpha2^{-/-}$ mice which lack α2 $GABA_AR$ specifically from spinal and peripheral sites, were treated systemically with HZ166, a novel BDZ site agonist with reduced sedative properties. Specific ablation of α2 $GABA_AR$ from all spinal and peripheral sites completely abolished the α2 mediated component of HZ166-induced antihyperalgesia in a neuropathic pain model, indicating that α2 $GABA_AR$ in the brain did neither contribute to or influence HZ166-induced antihyperalgesia. These results also rule out indirect antihyperalgesic effects resulting e.g. from central anxiolytic actions and a reversal of anxiety-induced hyperalgesia.

Results from project 1 and 2 provide clear evidence that at least the α2 mediated component of $GABA_AR$-induced antihyperalgesia results from a direct interaction with spinal nociceptive circuits and does not involve secondary effects originating from higher CNS areas. The third project therefore addressed the molecular organization of $GABA_AR$ subtypes in nociceptive circuits of spinal dorsal horn. Multiple-labeling experiments that combined different markers and very sensitive immunofluorescence assays revealed a highly organized $GABA_AR$ subtype distribution with α2 and α3 $GABA_AR$ being strategically located at excitatory and inhibitory interneurons and nociceptors terminals in the superficial dorsal horn laminae I-III. This study revealed $GABA_AR$ α2 , α3 and α5 subtypes localized to specific presynaptic and postsynaptic terminals and thus to sites critically involved in the GABAergic control of nociception. In summary, the combination of highly sophisticated genetic mouse models and of novel pharmacological tools together with anatomical, electrophysiological and behavioral approaches significantly extends the rational basis for the development of subtype-selective $GABA_AR$ ligands as novel spinally acting antihyperalgesic agents.

II Zusammenfassung

Chronische Schmerzen sind ein weitverbreitetes neurologisches Leiden, das wesentlich auf Veränderungen nociceptiver Schaltkreise im peripheren und zentralen Nervensystem beruht. Chronische Schmerzen treten häufig als Folge von Gewebeschädigung, chronischen Entzündungen oder Nervenschädigungen auf. Verminderte synaptische Hemmung im Hinterhorn des Rückenmarks trägt wesentlich zur Entstehung chronischer Schmerzen bei. Klassische Benzodiazepine (BDZ) verstärken synaptische Hemmung durch positive allosterische Modulation von $GABA_A$-Rezeptoren ($GABA_AR$). Ihre spinale Applikation wirkt in Nagern und im Menschen antihyperalgetisch, nach systemischer Gabe bewirken sie jedoch zumindest beim Menschen keine generelle Schmerzhemmung. Zudem ist insbesondere die langfristige Anwendung klassischer BDZ durch Nebenwirkungen wie Sedierung, Einschränkung der Gedächtnisleistung, Toleranzentwicklung und Abhängigkeit wesentlich eingeschränkt. $GABA_AR$ existieren in verschiedenen Subtypen, die sich in ihren funktionellen und pharmakologischen Eigenschaften unterscheiden. Sie werden typischerweise anhand ihrer α Untereinheit klassifiziert. Die antihyperalgetische Benzodiazepin-Wirkung wird hauptsächlich durch $GABA_AR$ vermittelt, die α2 bzw. α3 Untereinheiten enthalten (α2 bzw. α3 $GABA_AR$). Die meisten unerwünschten Effekte werden hingegen von supraspinalen α1 $GABA_AR$ vermittelt. Neue Subtyp-spezifische $GABA_AR$-Liganden könnten demnach schmerzlindernde Eigenschaften haben, ohne die typischen Nebenwirkungen klassischer BDZ aufzuweisen. Um dieses Konzept weiter zu untermauern, wurden in der vorliegenden Arbeit die an der spinalen Naociception beteiligten GABAergen Netzwerke einer vertieften Analyse unterzogen.

Im Rückenmark sind $GABA_AR$ sowohl postsynaptisch auf intrinsischen Neuronen des Hinterhorns als auch auf den Terminalen primär afferenter Nozizeptoren exprimiert, wo sie durch primärafferente Depolarisation (engl. primary afferent depolrization, PAD) zur präsynaptischen Hemmung beitragen. Die meisten dieser präsynaptischen $GABA_AR$ gehören zum α2 Typ. Im ersten Teilprojekt der vorliegenden Dissertation untersuchten wir Mäuse, deren Nozizeptoren entweder keine α2 $GABA_AR$ exprimierten (*sns*-$\alpha2^{-/-}$) oder aufgrund einer Punktmutation insensitiv gegenüber Diazepam waren (*sns*-$\alpha2^{R/-}$). Basale Schmerzschwellen sowie entzündliche und neuropathische Neuropathie waren in

beiden Mausstämmen unverändert. Im Entzündungsschmerzmodell war jedoch der antihyperalgetische Effekt von intrathekal injiziertem Diazepam signifikant vermindert.

Im zweiten Teilprojekt sollte der mögliche Beitrag supraspinaler α2 $GABA_AR$ zur Antihyperalgesie untersucht werden. Ein solcher Beitrag könnte etwa in einer Aufhebung angstvermittelter Hyperalgesie oder in einem genuinen antihyperalgetischen Effekt bestehen. Hierzu wurden *HoxB8*-$\alpha2^{-/-}$ Mäuse, denen α2 $GABA_AR$ im Rückenmark und in der Peripherie fehlen, systemisch mit HZ166 behandelt. HZ166 ist ein neu entwickelter Agonist an der Benzodiazepin-Bindungsstelle von $GABA_AR$ mit reduzierten sedativen Eigenschaften. Die selektive Ablation spinaler und peripherer α2 $GABA_AR$ führte zu einem kompletten Verlust der α2-vermittelten Komponente der HZ166-induzierten Antihyperalgesie im Neuropathiemodell. Diese Ergebnisse zeigen, dass supraspinale α2 $GABA_AR$ nicht wesentlich zur HZ166-vermittlelten Antihyperalgesie beitragen, und legen damit auch nahe, dass indirekte Effekte, die z.B. durch die anxiolytische Wirkung zustande kommen könnten, keine wesentliche Rolle spielen.

Im dritten Teilprojekt wurde daher die molekulare Zusammensetzung der $GABA_AR$-Subtypen im Hinterhorn des Rückenmarks einer detaillierten Analyse unterzogen. Histologische Färbung von verschiedenen Markerproteinen im selben Schnitt und hochsensitive Detektionsmethoden zeigten, dass die Verteilung der $GABA_AR$ im Hinterhorn des Rückenmarks einen sehr hohen Organisationgrad aufweist. Insbesondere α2 und α3 $GABA_AR$ zeigten eine strategisch äusserst effektive Verteilung auf Nociceptorterminalen und auf exzitatorischen und inhibitorischen Interneuronen. Die Ergebnisse dieser Untersuchungen belegen, dass α2, α3 und α5 $GABA_AR$ auf prä- und postsynaptischen Kompartimenten spezifischer Neuronentypen lokalisiert sind, die eine zentrale Rolle für die GABAerge Kontrolle der Nociception spielen.

Zusammenfassend konnte in dieser Arbeit durch die Kombination von transgenen Mausmodellen mit neu entwickelten GABAergen Wirkstoffen und mit Hilfe von anatomischen, elektrophysiologischen und verhaltensbasierten Methoden die neurobiologische Basis für Entwicklung von Subtyp-spezifischen $GABA_AR$-Liganden mit antihyperalgetischen Eigenschaften deutlich erweitert werden.

INTRODUCTION

Introduction

1.1 Pain

The survival and welfare of higher organisms critically depend on the ability to detect noxious environmental factors and to respond properly. Exposure to noxious stimuli evokes a protective response action resulting in the immediate withdrawal from the stimulus. Conversely, humans who suffer from congenital insensitivity to pain have a reduced life expectancy due to recurrent trauma and infections (Cox et al., 2006). Acute pain hence serves an important protective function. However, when pain persists beyond the actual threat, it severely interferes with the wellbeing of organisms.

The International Association for the Study of Pain (www.iasp-pain.org) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". The prevalence of chronic pain and pain-related impairments were surveyed in European (Breivik et al., 2006), American (www.theacpa.org) and Canadian (Reitsma et al., 2011) populations. These studies indicated that chronic pain represents a challenging and debilitating condition contributing to major medical and socio-economic problems; it has even been suggested that relief from pain should be recognized as a human right (IASP, 2010).

Chronic pain is primarily distinguished from acute pain by its long duration. It can be further sub-classified based on its spatial and temporal pattern, its intensity and etiology. On this basis, a detailed classification of chronic pain has been established by the IASP (Merskey and Bogduk, 1994). Chronic pain often involves plasticity in the nociceptive stimuli processing that can occur at all levels of the neuraxis. The complexity of these changes is probably a major reason for the difficulties in the management of chronic pain syndromes. Typical symptoms of chronic pain include hyperalgesia (increased pain to noxious stimuli), allodynia (pain due to a stimulus which does not normally provoke pain) and spontaneous pain (pain felt in the absence of sensory stimulation). Hyperalgesia occurring at the site of injury is called primary hyperalgesia, while enhanced pain sensitivity in uninjured healthy tissue is termed secondary hyperalgesia. Both peripheral and central components can contribute to primary hyperalgesia and probably also to spontaneous pain, whereas secondary hyperalgesia and allodynia originate exclusively from central sensitization. On the basis of its pathophysiology, pain

can be classified into (1) nociceptive (transient pain in response to noxious stimulus), (2) inflammatory (initiated by tissue damage and/or inflammation), (3) neuropathic (caused by a primary lesion in the nervous system), and (4) dysfunctional pain (hypersensitivity to pain resulting from abnormal central processing of normal input) (Woolf, 2010).

While our knowledge of the molecular and cellular mechanisms of acute pain has increased significantly during the last decades, our understanding of the mechanisms of chronic pain is still incomplete. Accordingly, chronic pain treatment, which still relies mainly on opioids and cyclooxygenase inhibitors, is very often insufficient. Both families of analgesics lack efficacy particularly in neuropathic pain and often result in major side effects when taken for prolonged periods of time. Therefore, novel therapeutic approaches are required. A large number of genetic, electrophysiological, neuroanatomical and pharmacological studies are currently undertaken to elucidate the molecular mechanisms that underlie chronic pain with the ultimate aim to improve pain treatment.

1.2 Nociception

According to IASP, nociception is defined as "The neural processes of encoding and processing of noxious stimuli"(Loeser and Treede, 2008). It has three components: (1) peripheral, where the afferent activity is initiated by a subpopulation of peripheral nerve fibers called nociceptors, (2) spinal, where most of the nociceptors terminate and transmit signals to second order neurons and (3) supraspinal, where neural processes result in conscious pain perception. These pathways are schematically represented below (fig. 1).

1.2.1 Peripheral Components of Nociception

Nociceptors are sensory nerve cells activated by mechanical, thermal or chemical stimuli that reach the noxious stimulation strength range. They have peripheral and central axonal branches that innervate their target organs (skin, visceral organs and skeletal and cardiac muscle) and the spinal cord or brain stem, respectively. The cell bodies of the nociceptors innervating the head reside in trigeminal ganglia (TG), while those of nociceptors innervating the rest of the body are located in the dorsal root ganglia (DRG). Upon exposure to noxious stimuli, nociceptors produce inward (depolarizing) currents within their peripheral terminals, which evoke action potentials (AP) when the

depolarizations become suprathreshold. These signals are then transmitted to second order neurons located in the ipsilateral trigeminal nucleus or the spinal dorsal horn. Nociceptors release L-glutamate from their central terminals to excite second order dorsal horn neurons.

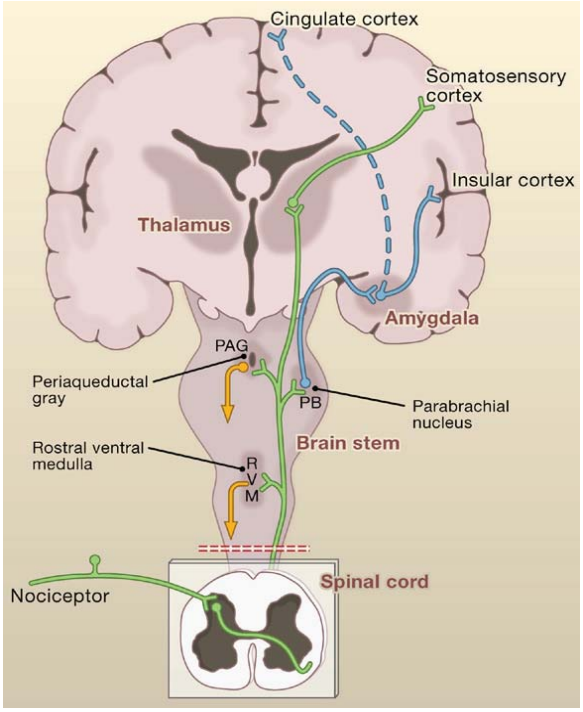


Fig. 1. Schematic of afferent pathways underlying the sensation of pain. Activated nociceptors convey noxious information to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons transmits information about the location and intensity to the somatosensory cortex via the thalamus. Other projection neurons engage the cingulate and insular cortices via connections in the brainstem (parabrachial nucleus) and amygdala, contributing to the affective component of the pain experience. This ascending information also accesses neurons of the rostral ventral medulla and midbrain periaqueductal gray to engage descending feedback systems that regulate the output from the spinal cord. Reproduced from Basbaum et al. (2009).

Based on their conduction velocity, nociceptors are categorized into two classes: (1) medium diameter myelinated Aδ afferents that mediate acute, sharp and well-localized fast pain and (2) small diameter unmyelinated C fibers that convey poorly localized, prolonged, dull and burning slow pain sensation. The latter are further subdivided into peptidergic and non-peptidergic nociceptors. The former release neuropeptides (substance P and calcitonin-gene related peptide, CGRP), in addition to glutamate. These neuropeptides are absent in non-peptidergic nociceptors. Release of CGRP and substance P from the peripheral terminals causes vasodilation and the so called flare response (Basbaum et al., 2009). Peptidergic and non-peptidergic fibers not only differ in their neuropeptide content but also in other neuroanatomical and molecular characteristics (Snider and McMahon, 1998). Peptidergic nociceptors express the TrkA neurotrophin receptor, which responds to nerve growth factor (NGF). Non-peptidergic fibers produce chondroitin sulfate proteoglycans and express the c-Ret neurotrophin receptor targeted by glial-derived neurotrophic factor (GDNF), neurturin and artemin.

1.2.2 Spinal Components of Nociceptive Regulation

1.2.2.A Nociceptor Terminals in the Spinal Dorsal Horn

The central axons of nociceptors terminate in various laminae of the spinal dorsal horn with a specific distribution pattern (Rexed, 1952). Mechanoreceptive myelinated primary afferent fibers arborize in an area extending from lamina III–V, whereas nociceptive C afferents innervate lamina I – II. Recent studies have identified a group of cooling-specific C afferents that terminate in lamina I (Dhaka et al., 2008) and two possible candidates for low-threshold mechanoreceptive C fibers that also project to lamina II, (Liu et al., 2007; Seal et al., 2009). The outer half of lamina II (lamina IIo) distinctly harbors peptidergic fiber terminals whereas the inner half (lamina IIi) contains non-peptidergic terminals (Hunt and Mantyh, 2001; Zylka, 2005) (fig. 2). The terminals of unmyelinated and myelinated primary afferents form the central axons of type I and type II synaptic glomeruli respectively, as shown by early ultrastructural studies (Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva et al., 1985). In lamina II, the densities of type I and type II synaptic glomeruli are 2.58 and 0.32. Within central lamina II region, around 79% of glomeruli are of type I whereas in the ventral lamina II region approximately 66% are of type II.

Recent studies have explored the possibility of distinct pain modalities being conveyed by specific classes of nociceptors hosting specific molecules (Abrahamsen et al., 2008). Scherrer *et al* (2009) analyzed the differential distribution of μ- and δ-opioid receptors in distinct populations of peptidergic and non-peptidergic nociceptors, respectively, and showed that they control either thermal or mechanical pain. Similarly, the ablation of Mas-related G-protein-coupled receptor member D (MRGPRD)-containing nonpeptidergic fibers resulted in a selective loss of sensitivity to noxious mechanical (but not thermal) stimuli (Cavanaugh et al., 2009). Recently, the expression of transient receptor potential vanilloid-1 (TRPV-1) receptors, critical for the development of thermal hyperalgesia (Caterina et al., 2000), has been found to be restricted to a subset of peptidergic nociceptors (Cavanaugh et al., 2009). Mechanical nociception depends on the Nav1.8 nociceptors expressing the vesicular glutamate transporter VGluT2 (Lagerström et al., 2011).

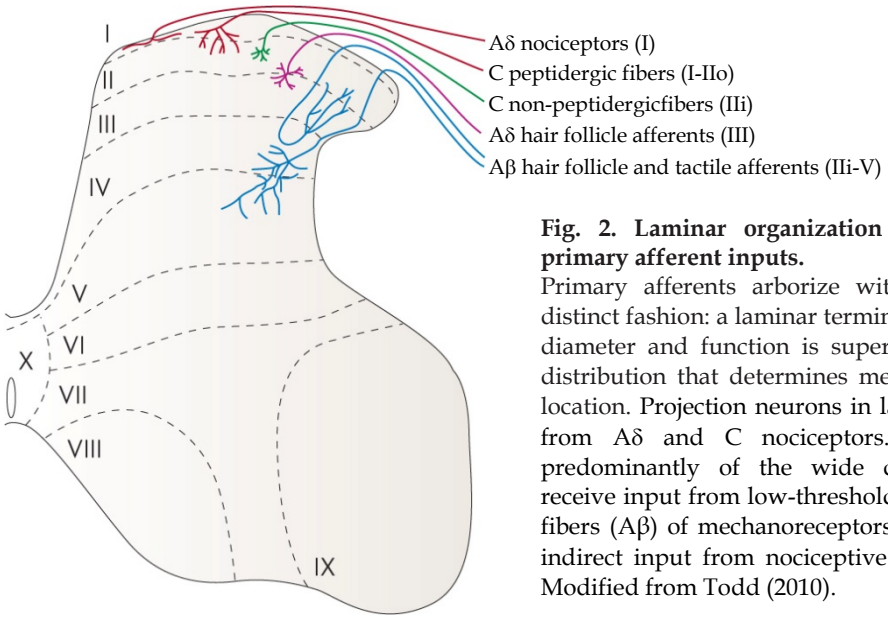


Fig. 2. Laminar organization of the dorsal horn and primary afferent inputs.
Primary afferents arborize within the dorsal horn in a distinct fashion: a laminar termination pattern based on fiber diameter and function is superimposed on a somatotopic distribution that determines mediolateral and rostrocaudal location. Projection neurons in lamina I receive direct input from Aδ and C nociceptors. Lamina V neurons are predominantly of the wide dynamic-range type. They receive input from low-threshold large-diameter myelinated fibers (Aβ) of mechanoreceptors as well as both direct and indirect input from nociceptive afferent fibers (Aδ and C) Modified from Todd (2010).

1.2.2.B Interneurons

Virtually all neurons in lamina II and most of those in lamina I (excluding the projection neurons) and lamina III have axons that remain in the spinal cord and arborize locally (thus called interneurons). They can be divided into two main classes: 1) excitatory (glutamatergic) and 2) inhibitory (GABAergic and/or glycinergic). Excitatory interneuron terminals in the dorsal horn can be identified by the presence of VGLUT2 (Todd et al., 2003; Maxwell et al., 2007; Yasaka et al., 2007). VGLUT2-containing boutons are numerous in laminae I–III, most of which originate from local excitatory interneurons. About 25%, 30% and 40% of neurons are GABAergic in laminae I, II and III, respectively (Polgar et al., 2003), a subpopulation of those is, in addition, glycinergic. Very few neurons have been found to be only glycinergic and not GABAergic (Todd and Sullivan, 1990). Nevertheless, electrophysiological studies have identified synapses in lamina I-III that are purely glycinergic (Keller et al., 2001; Yasaka et al., 2007). The axons of inhibitory interneurons can be identified with antibodies against the vesicular inhibitory amino acid transporter (VIAAT, which transports GABA and glycine into presynaptic vesicles), glutamate decarboxylase (GAD, the GABA-synthesizing enzymes) or the glycine transporter type 2 (GLYT2, which transports glycine into presynaptic terminals).

In some respects, the inhibitory action of GABA in the spinal cord is more complex than in many parts of the brain (see section 1.5.7). In brain, postsynaptic $GABA_AR$ activation reduces neuronal excitability primarily through hyperpolarization and the activation of a shunting conductance, and presynaptic $GABA_AR$ activation facilitates transmitter release and LTP induction (Ruiz et al., 2010). In the spinal cord, $GABA_AR$ on the terminals of primary afferent fibers contribute to presynaptic inhibition through primary afferent depolarization (Eccles et al., 1961). Primary nociceptors exhibit a high expression of sodium-potassium-chloride transporter NKCC1 (transports chloride, sodium and potassium into the cell) along with a low to nil expression of potassium chloride co-transporter KCC2 (extrudes chloride and potassium out of the cell) (Rivera et al., 1999; Sung et al., 2000) giving rise to sub-threshold primary afferent depolarization (PAD). Bicuculline-sensitive GABAergic inhibition has a major role in creating PAD (Nishi et al., 1974; Mokha et al., 1983). Activity-related increases in the concentration of glutamate (Rees et al., 1995) and potassium (Heinemann et al., 1990) also contribute to PAD. PAD inhibits transmitter release from the primary afferent terminal possibly through inactivation of voltage-gated Ca^{2+} and/or Na^{+} channels or through activation of a shunting conductance (Kullmann et al., 2005). Under certain conditions, PAD may become suprathreshold and would then facilitate chronic pain through the so called dorsal root potentials resulting in hyperalgesia and allodynia (Willis Jr, 1999).

Lamina II interneurons can also be classified based on the morphology of their dendritic trees. Most authors distinguish islet, central, vertical and radial cells (Grudt and Perl, 2002; Lu and Perl, 2003; Hantman et al., 2004), but a generally accepted classification that covers all interneurons in laminae I–III does still not exist. Furthermore, these morphologically defined groups do not represent homogeneous functional subpopulations (Todd, 2010).

1.2.2.C Projection Neurons

Dorsal horn projection neurons have axons that cross the midline, project rostrally, run in the contralateral white matter, and terminate in various brain stem and thalamic nuclei. They are mainly concentrated in lamina I but exist also in all other dorsal horn laminae, at least at lumbar levels. Quantitative retrograde tracing studies in the rat suggest that in the fourth lumbar segment (L4), projection neurons constitute ~5% of lamina I neurons (Spike et al., 2003). Attempts to classify projection neurons into discrete

categories were not successful yet. The neurokinin 1 receptor (NK1R)-containing lamina I neurons have been extensively studied because of their relevance in nociception (fig. 3). NK1R is the main target of substance P and is in the dorsal horn selectively expressed in neurons that respond to noxious stimuli (Salter and Henry, 1991). The ablation of these projection neurons prevents the development of hyperalgesia in inflammatory and neuropathic pain models (Mantyh et al., 1997; Nichols et al., 1999).

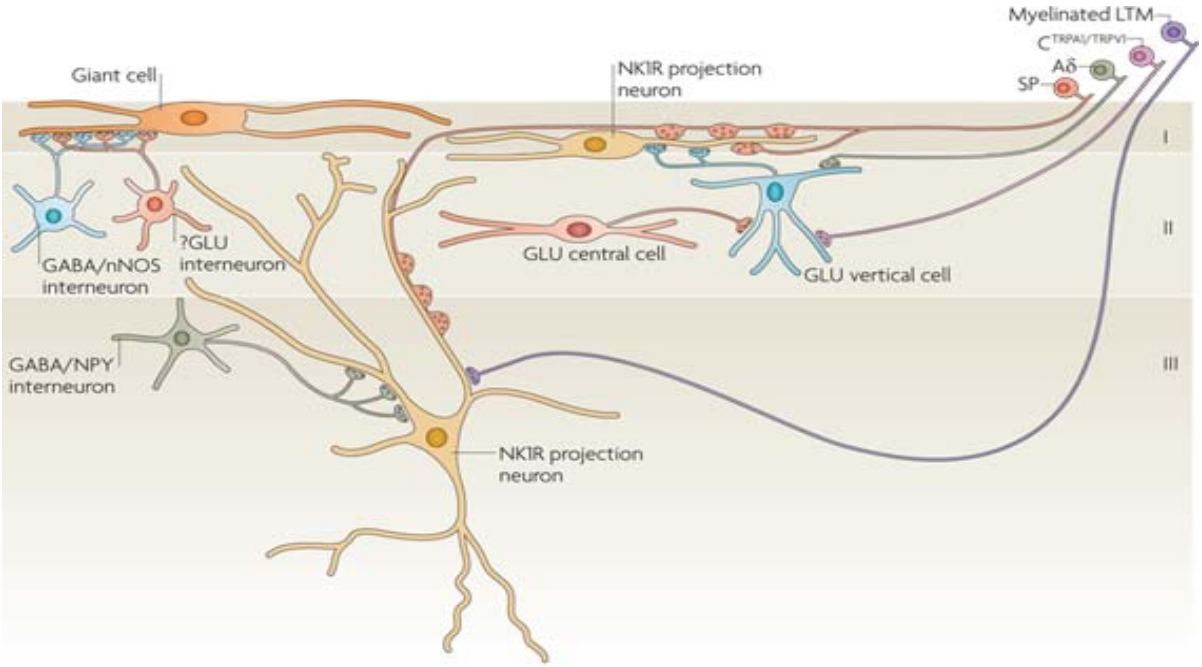


Fig. 3. Synaptic circuits in the superficial dorsal horn laminae I-III. Two NKIR-positive projection neurons in laminae I and III are densely innervated by substance P-containing primary afferents (SP). Additionally, the lamina III neurons receive an input from myelinated low threshold mechanoreceptive (LTM) afferents and GABAergic interneurons that contain neuropeptide Y (GABA/NPY interneuron). A giant lamina I neuron receive a substantial input from inhibitory GABA/nNOS interneuron. Lamina I projection neurons receive high density innervations from two glutamatergic interneuronal populations (Unknown subtype containing VGLUT2 (?GLU interneuron) and GLU vertical cells). GLU vertical cell are innervated by glutamatergic central cells (GLU central cell) and Aδ and C fibers expressing TRPA1 and TRPV1. Reproduced from Todd (2010).

1.2.2.D Descending Inputs

Serotonergic and noradrenergic systems contribute to descending monaminergic pathways projecting to the dorsal horn. Serotonergic pathways originate mainly from the nucleus raphe magnus whereas noradrenergic projections descend from the locus coeruleus. Axons of both systems terminate diffusely throughout the dorsal horn, and although some form synapses, much of their action is mediated through volume transmission (Zoli et al., 1999). Another descending pathway that may have a role in spinal pain control consists of GABAergic axons that descend from the rostral ventromedial medulla, arborize widely in the dorsal horn (Antal et al., 1996; Kato et al., 2006) and synapse with lamina II interneurons (Kato et al., 2006). The consequences of

activation of monaminergic neurons are determined largely by the distribution of α1 and α2 adrenergic receptors and 5-hydroxytryptamine (5-HT; serotonin) 1A (5HT1A) and 5HT3 receptors. Most neurons hyperpolarize in response to both monamines but some islet and central cells are depolarized by 5-HT, and some GABAergic neurons (including islet cells) are depolarized by noradrenaline (Abe et al., 2009; Gassner et al., 2009). Additionally, monamines can suppress primary afferent inputs to superficial dorsal horn neurons through a presynaptic mechanism (Lu et al., 2002).

1.2.3 Supraspinal Components of Nociception

Anterograde tracing experiments from the spinal dorsal horn have shown that ascending projections terminate in the caudal ventrolateral medulla (CVLM), the nucleus of the solitary tract (NTS), the lateral parabrachial area (LPb), the periaqueductal grey matter (PAG) and certain nuclei in the thalamus (Gauriau and Bernard, 2004). Extensive collateralization of axons, with some lamina I neurons projecting to at least three targets (e.g., LPb, PAG and thalamus) has also been observed (Al Khater and Todd, 2009). Most of the projection neurons have only contralateral projections, but some exhibit bilateral projections, as well (Spike et al., 2003). Quantitative retrograde tracing studies in the rat indicated that 95% of all lamina I projection neurons project to the LPb, around a third to the PAG, a quarter to the NTS and <5% to the thalamus (Spike et al., 2003). Thus, the low number of lamina I spinothalamic cells seem to give raise to pathways essential for conscious pain perception. The perception of pain involves sensory-discriminative, affective-motivational and cognitive-evaluative dimensions represented in distinct pain pathways and brain regions activated during the experience of pain (pain matrix). The primary (S1) and secondary (S2) somatosensory cortices that receive input from the ventrolateral thalamic nuclei constitute the lateral pain system. This system affects the sensory and discriminative components (location and intensity) of pain. The medial pain system branches at the level of the medulla and ascends via the medial thalamus to hypothalamic nuclei, limbic regions (mainly anterior cingulate cortex (ACC)), the insular cortex (IC) and prefrontal areas. These regions control emotion, arousal and attention. This medial pain pathway is therefore proposed to mediate the unpleasant, affective imensions of pain and the motivation to escape from the noxious event (Treede et al., 1999; Price, 2000; Brooks and Tracey, 2005; Bushnell and Apkarian, 2006).

1.3 Animal Pain Models

Most of our knowledge of the molecular mechanisms of acute nociceptive and pathological pain has been obtained from experiments in animal models of pain.

Acute nociceptive pain

Classical tests of nociception in rodents involve the quantification of sensitivities to noxious heat, punctate mechanical stimuli, pressure and chemical activators of nociceptors. Frequently used tests include the tail flick test, the hot plate test and the plantar (Hargreaves et al., 1988) test. In the tail flick and plantar test a radiant heat stimulus of pre-defined intensity is directed onto the tail or plantar surface of one hindpaw, respectively. In the hot plate test, the animal is placed onto a heated surface. In all these tests, the typical read-out is the time interval (latency) between the application of the noxious stimulus and a withdrawal or escape response of the animal. One advantage of the plantar test is that it allows the comparison of responses elicited in inflamed and non-inflamed paws.

Sensitivity to punctate mechanical stimuli is typically measured with calibrated von Frey filaments which are applied on the plantar surface of one hind paw and which exert a defined pressure upon bending. More recently developed versions include force transducers (electronic von Fey filaments) or filaments which are moved at a pre-defined and electronically-controlled speed towards the animal paw (dynamic von Frey filaments). In all these tests, sensitivity is measured as the threshold force at which an animal withdraws the stimulated paw.

Chemical sensitivity is typically assessed by injecting compounds, which directly activate nociceptor terminals. Such compounds include the TRPV1 agonist capsaicin, the TRPA1 agonist mustard oil, or formalin, which at low concentrations is also a selective activator of TRPA1. The animal's sensitivity is measured in most cases by counting the number of nociceptive responses e.g. of flexor responses (so called flinches).

Hyperalgesia and allodynia

In mice, inflammatory pain can be generated by injecting pro-inflammatory agents (e.g., complete Freund's adjuvant, zymosan A, carrageenan) into one hind paw (Meller and

Gebhart, 1997). All these agents induce a robust heat and mechanical hyperalgesia of the inflamed paw within hours.

Neuropathic pain can be induced by peripheral nerve injuries, injections of neurotoxic drugs or by inducing diabetic syndrome. The most frequently used models involve partial mechanical injuries of peripheral nerves.

In the chronic constriction injury model (CCI model; Bennett and Xie, 1988; Sugimoto et al., 1990) a partial nerve damage is induced through loose ligations put around the sciatic nerve. The constriction results from intraneural edema within the nerve leading to a preferential loss of myelinated afferents. Mice exposed to CCI develop robust mechanical and heat hyperalgesia, and cold allodynia. These symptoms develop over the first post-operative week, peak during the second week, and gradually disappear by around 4-5 weeks.

In the spinal nerve ligation (SNL; Kim and Chung, 1992) model, tight ligatures are placed around the L5 and L6 spinal nerves before these join the lumbosacral plexus. Animals exposed to SNL rapidly develop signs of tactile allodynia, which appear within the first post-operative day and last for several months. In addition, they show a thermal hyperalgesia that starts during the first week and lasts for around 1 month.

In the spared nerve injury (SNI) model two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) are ligated and cut, leaving only the sural nerve intact (Decosterd and Woolf, 2000). Like the SNL model, this also results in a rapidly developing and prolonged tactile and cold allodynia in the affected hind limb, and comparatively minor increases in heat sensitivity.

Mechanical sensitization in neuropathy manifests not only in increased responses to punctate mechanical stimuli (static allodynia) and pressure, but also in dynamic allodynia which can be tested by stroking an animal's flank with a paint brush.

1.4. Mechanisms of Hyperalgesia and Allodynia

Similar to many other neurological diseases such as epilepsy, sleep disorders and anxiety, pathological pain is believed to originate from an imbalance of excitatory and inhibitory drive. Examples for increase excitation in spinal dorsal horn include short term and long term increases in the strength of synaptic transmission between primary nociceptors and dorsal horn second order neurons. Repetitive stimulation of C fibers leads to a transient homosynaptic activity-dependent progressive enhancement of the postsynaptic potentials, which results in an increased excitability of dorsal horn neurons called wind up (Mendell, 1984; Herrero et al., 2000). This phenomenon results in progressive increase in pain sensitivity to repetitive noxious heat or mechanical stimuli (Price et al., 1977; Staud et al., 2003). A prolonged form of homosynaptic activity-dependent plasticity (long term potentiation; LTP) has been identified at the excitatory synapses between C fibers and NK1R-expressing lamina I projection neurons. Both high and low frequency stimulation can induce LTP in these neurons probably through Ca^{2+}-dependent postsynaptic mechanisms (Ikeda et al., 2003; Ikeda et al., 2006). As NK1R-expressing projection neurons have an important role in the development of chronic pain states, LTP of their nociceptive inputs is likely a major mechanism of hyperalgesia (Mantyh et al., 1997; Nichols et al., 1999; Sandkühler, 2009).

Facilitated transmission between intrinsic excitatory dorsal horn neurons may enhance activation of lamina I projection cells through polysynaptic pathways. This mechanism has been shown to involve phosphorylation of Kv4.2 (at position S616), a downstream target for phosphorylated extracellular signal-regulated kinases (ERKs), expressed in excitatory interneurons in lamina II. This phosphorylation results in a reduction of A-type potassium currents, leading to an increase in excitability (Hu et al, 2006). Since ERKs are activated in many neurons following noxious stimulation, this mechanism could also contribute to hyperalgesia (Todd, 2010; Yasaka et al., 2010).

An imbalance of excitatory and inhibitory drive can also be caused by diminished inhibition. This concept dates back to the gate control theory of pain (Melzack and Wall, 1965), which proposed that inhibitory interneurons would regulate the strength of nociceptive inputs transmitted from the periphery to higher CNS areas through the spinal cord. It has been shown that spinal pharmacological antagonism of $GABA_A$ and

glycine receptors with bicuculline or strychnine induces tactile allodynia and hyperalgesia (Beyer et al., 1985; Roberts et al., 1986) and intrathecal application of GABA reversed thermal and mechanical sensitivity in rats with chronic constriction nerve injury (Eaton et al., 1999). Many studies from different groups published in last decade have shown that a reduction in dorsal horn synaptic inhibition occurs also endogenously in the natural course of inflammatory and neuropathic diseases (Zeilhofer and Zeilhofer, 2008).

1.4.1 Spinal Disinhibition in Inflammatory Hyperalgesia

Inflammation is a complex defense reaction orchestrated by components of the innate and acquired immune system. Many mediators, including bradykinin, substance P, ATP, prostaglandins (mainly PGE2 and PGI2), growth factors (e.g., NGF), proteases, protons, nitric oxide (NO), pro-inflammatory cytokines and chemokines contribute to an increased excitability of nociceptors. These algogens activate three classes of receptors: (1) G-protein coupled receptors for prostaglandin (EP2, EP4, IP and DP1) and histamine (H1); (2) Tyrosine kinase receptors (TrkA and TrkB); (3) ligand gated ion channels (TRPV and P_2X receptors. For detailed review see (Linley et al., 2010).

Peripheral inflammation induces secondary changes in the CNS, particularly in the spinal dorsal horn. The following example illustrates how signaling cascades are interlinked to alter neuronal excitability. Inflammation causes an increase in the expression of cyclooxygenase-2 (COX-2) and inducible microsomal prostaglandin E synthase 1 (mPGES-1), enzymes required for prostaglandin E2 (PGE2) synthesis (Beiche et al., 1996; Murakami et al., 2000; Samad et al., 2001; Claveau et al., 2003). The resulting increased EP2 receptor activation stimulates G_s/PKA pathway leading to the PKA phosphorylation and inhibition of α3 subunit containing glycine receptors (GlyRα3). These receptors are densely expressed in the superficial laminae concentrating in lamina IIi (Harvey et al., 2004). PGE2 thus reduces glycinergic transmission in the majority of superficial dorsal horn neurons resulting in central inflammatory hyperalgesia (Ahmadi et al., 2002; Reinold et al., 2005) (fig. 4A). Mice lacking either EP2-receptor or GlyRα3 or carrying a mutated PKA regulatory subunit exhibit strongly reduced inflammatory pain indicating a near absence of pro-nociceptive actions of PGE2 (Harvey et al., 2004; Reinold et al., 2005; Hösl et al., 2006). Interestingly, the development of neuropathic pain in CCI model is not altered in these mice (Hösl et al., 2006).

1.4.2 Spinal Disinhibition in Neuropathic Hyperalgesia

Mechanisms of disinhibition resulting in neuropathic pain have been extensively in rodents. A reduction in the proportion of lamina II neurons exhibiting primary afferent-evoked inhibitory postsynaptic currents has been detected (Moore et al., 2002), supporting the view that disinhibition contributes to neuropathic pain symptoms. Earlier studies have reported an almost complete loss of GABA-immunoreactivity in dorsal horn neurons at around two weeks after CCI (Ibuki et al., 1996; Eaton et al., 1998). Others have, in addition, found that GAD-65 expression was down-regulated after CCI or SNI (Moore et al., 2002). An apoptotic cell death of GABAergic interneurons has been proposed as an underlying mechanism (Moore et al., 2002), but more recent evidence suggests that CCI does not cause a loss of neurons in the dorsal horn and does not alter the portion of GABA- or glycine-immunoreactive neurons (Polgar et al., 2003; Polgar et al., 2004). Alternative explanations of reduced inhibitory control after peripheral nerve injury include a depletion of GABA (or glycine) from presynaptic terminals. However, following SNI, the level of GABA in GABAergic boutons in lamina I-III and the number of $GABA_AR$ were similar in neuropathic and intact sides (Polgár et al., 2008). The possibility of disinhibition by the reduction of the number of $GABA_AR$ has also been investigated, but the number of $GABA_AR$ was not altered in neuropathic mice (Moore et al., 2002, Polgar et al., 2008).

Peripheral nerve injury leads to microglia-dependent central sensitization and this pathway, leading to diminished synaptic inhibition, has been recognized as a key event in the generation of neuropathic pain following SNI (Tsuda et al., 2003; Scholz and Woolf, 2007). The damage of nerve fiber leads to the release of cytokine CCL2 that mediates microglial recruitment and activation. The activated microglial cells release brain derived neurotrophic factor (BDNF) and subsequently result in TrkB-mediated down-regulation of the potassium chloride co-transporter KCC2 in dorsal horn neurons (Coull et al., 2003; Coull et al., 2005) (fig. 4B). P2X family receptors are thought to be involved in the release of BDNF and are thus considered potential targets for novel analgesics (Chessell et al., 2005; Honore et al., 2005).

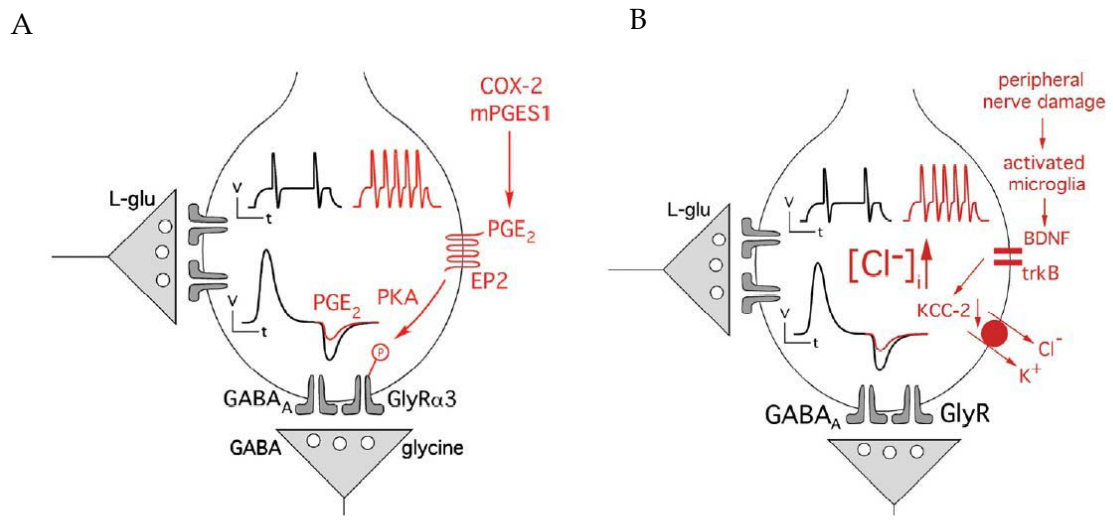


Fig. 4. Diminished synaptic GABAergic and glycinergic control. (A) Disinhibition in inflammatory hyperalgesia. Cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase (mPGES1) become induced in response to inflammation in peripheral tissues and produce PGE2 in the spinal cord. PGE2 binds to PGE2 receptors of the EP2 subtype, which increase cAMP levels and activate protein kinase A (PKA). PKA then phosphorylates and inhibits a specific subtype of glycine receptors containing the α3 subunit, which regulate the excitability of superficial dorsal horn neurons. This disinhibition facilitates the firing of these neurons and promotes transmission of nociceptive signals through the spinal cord to higher brain areas where pain becomes conscious. (B) Disinhibition in neuropathic pain states. Peripheral nerve damage activates spinal microglia which releases brain-derived neurotrophic factor (BDNF). BDNF subsequently down-regulates the potassium-chloride cotransporter (KCC-2), reducing the inhibitory action of $GABA_A$ and glycine receptors. Reproduced from Zeilhofer and Zeilhofer (2008).

1.4.3 Acute and Chronic Pain Treatment

At present the most frequently used drugs in chronic pain treatment are opioids and cyclooxygenase inhibitors. Opioids are very effective in the treatment of acute pain and of certain chronic pain states, in particular of cancer associated pain. They are however less effective (and less well tolerated) in patients with chronic inflammatory pain. Cyclooxygenase inhibitors on the other hand are highly effective against inflammatory pain, while both opioids and cyclooxygenase inhibitors are poorly effective in neuropathic pain patients (Malmberg and Yaksh, 1992; Lashbrook et al., 1999). Neuropathic pain is at present most frequently treated with anticonvulsive drugs, which either block voltage gated Na^+ channels (carbamazepine or oxcarbazepine; Ilyin et al., 2006) or modulate voltage gated Ca^{2+} channel (gabapentin and pregabalin; Tanabe et al., 2008). Both of these drug classes reduce neuronal excitability and synaptic transmission. The clinical efficacy of the more recently developed gabapentin and pregabalin has been proven in a large number of clinical studies, but their efficacy with numbers needed to treat (NNT) between 5 and 6 is still far from being optimal (Moore et al, 2011).

As outlined above, an alternative approach to neuropathic or chronic pain in general could be the strengthening of synaptic inhibition. Although gabapentin and pregabalin contain "gaba" in their names and gabapentin has even structural similarity with GABA, neither gabapentin nor pregabalin act on $GABA_AR$ (Coderre et al., 2005; Li et al., 2011) but bind instead to an accessory subunit of certain voltage-gated Ca^{2+} channels (Gee et al., 1996; Marais et al., 2001). The therapeutic option of increasing inhibition is therefore totally unexplored so far. The following chapters hence discuss $GABA_AR$ in general and specifically in the spinal cord.

1.5 GABA Receptors

In mammalian central nervous system, more than one third of synapses release the inhibitory neurotransmitter GABA that activates $GABA_AR$. These receptors are classified into two types based on their biochemical, pharmacological and physiological properties (Bowery et al., 1980): 1) The ionotropic $GABA_AR$, a member of the Cys-loop ligand-gated ion channel superfamily, which also includes nicotinic acetylcholine, glycine and 5-HT3 receptors and 2) the metabotropic $GABA_B$ receptors ($GABA_BR$), a member of the group C family of G-protein-coupled receptors including metabotropic glutamate receptors. $GABA_BR$ mediate slow inhibition of neuronal excitability through the opening of inwardly rectifying K^+ channels and the inhibition of voltage-gated calcium channels (VGCCs). They also inhibit adenylyl cyclase (Pan et al., 2008). $GABA_AR$ mediating fast synaptic inhibition are permeable to chloride (Cl^-) and to a lesser extent, to bicarbonate (HCO_3^-) (Farrant and Kaila, 2007). Increased inhibition by $GABA_AR$ causes behavioral changes, including sedation, anxiolysis, anterograde amnesia, muscle relaxation and increased seizure threshold.

1.5.1 $GABA_A$ Receptors

$GABA_AR$ are heteropentameric ion channels assembled from at least 19 subunits (α1 6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3). The most common subtype in the CNS is composed of two α, two β, and one γ subunit. Structurally, they are composed of a central pore for anion flux, two GABA binding sites (each formed by an α and β interface) and one BDZ -ligand binding site at the α and γ subunit interface (Möhler, 2006). $GABA_AR$ can be composed of varying combinations of different α subunits (fig. 5).

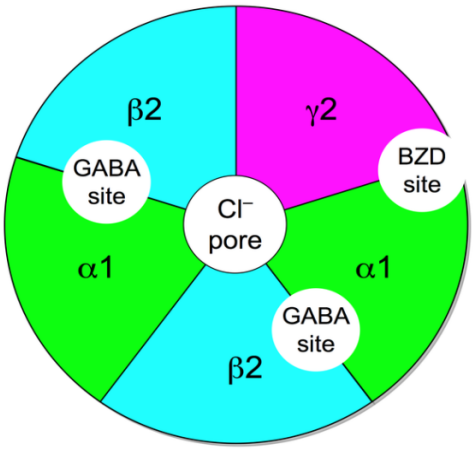


Fig. 5. $GABA_A$ receptor with its major ligand binding sites.
Schematic illustration of a $GABA_AR$ protein formed from five subunits $((\alpha1)_2\ (\beta2)_2\ (\gamma2))$. It also depicts chloride (Cl^-) ion channel pore, two GABA active binding sites at the α1 and β2 interfaces, and BDZ allosteric binding-site at the α1 and γ2 interface.

1.5.2 $GABA_A$ Receptor-Mediated Regulation of Neurotransmission

The first and foremost significant action of GABA in CNS is to decrease neuronal excitation by increasing the conductance for chloride ions. In neurons, depending on the intracellular anion concentration and the membrane potential, $GABA_AR$ can mediate chloride (Cl^-) current which is either hyperpolarizing (inward) or depolarizing (outward) and depolarizing HCO_3^- current. Activation of $GABA_AR$ by GABA and other agonists (such as muscimol) leads to an increase in Cl^- conductance driving the membrane potential towards the chloride equilibrium potential. Most adult CNS neurons have negative chloride equilibrium at around -65 mV. In these neurons $GABA_AR$ activation results in a decreased neuronal excitability thereby reducing the probability for an action potential in the postsynaptic neuron. However, excitatory effects of $GABA_AR$ activation by the net effluxes of Cl^- and K^+ ions have also been reported, notably during development (Ben-Ari et al., 1997; Taketo and Yoshioka, 2000), as well as in certain cell populations, such as some hippocampal interneurons (Lamsa and Taira, 2003) and in DRG neurons (see 1.2.3).

GABA mainly mediates phasic (transient) inhibition through the synaptically localized $GABA_AR$. These receptors are composed of α1 , α2 , α3 or α5 subunits in combination with the ubiquitous γ2-subunit that drives receptor clustering at the synapse (Essrich et al., 1998). Activation of extrasynaptic $GABA_AR$ by the GABA spilled over from the synaptic cleft has been shown to cause tonic (persistent) inhibition (Brickley et al., 1996). These extrasynaptic $GABA_AR$ contain α4 and α6 subunits as well as δ subunit. α5 $GABA_AR$ are found both at the synaptic and extrasynaptic sites. Tonic inhibition is

distinct from the transient activation of synaptic $GABA_AR$ that result in classical inhibitory postsynaptic currents and from the slow and transient response of the metabotropic $GABA_BR$. The generation of tonic inhibition upon activation of slowly desensitizing extrasynaptic receptors by low concentrations of GABA is attributed to the presence of the δ subunit (Haas and Macdonald, 1999; Bianchi et al., 2002). Recently extrasynaptic $GABA_AR$ have been identified as novel targets for endogenous and clinically relevant molecules such as neuroactive steroids (Lambert and Belelli, 2002), ethanol (Sundstrom-Poromaa et al., 2002), analgesics (Krogsgaard-Larsen et al., 2004) and anticonvulsant drugs (Cheng et al., 2006). Neurosteroids (e.g., the progesterone metabolite allopregnanolone) have been shown to specifically modulate neuronal excitability through endocrine, paracrine, or autocrine actions at extrasynaptic $GABA_AR$ in neurons (Belelli et al., 2005).

$GABA_AR$ contain several allosteric binding sites for multiple ligands, in particular BDZ - site ligands, barbiturates, ethanol, neuroactive steroids, volatile and intravenous anesthetics. Classical BDZ are positive allosteric modulators of $GABA_AR$ and enhance the apparent affinity of GABA, whereas inverse agonists (e.g., Ro 15-4513) have the opposite effect. The action of both agonists and inverse agonists can be blocked by the antagonist flumazenil, which lacks intrinsic activity (Hunkeler et al., 1981; Möhler, 2006).

1.5.3 Functional Analysis of $GABA_A$ Receptors Using *In vitro* and *In vivo* Tools

In the last two decades, the functional significance of the molecular diversity of $GABA_AR$ has been unraveled by novel genetic and pharmacological tools employed to study the functions of specific $GABA_AR$ isoforms *in vitro* and *in vivo*. $GABA_AR$ subunit specific knock-out mice were generated, but the applicability of these mice in studies to understand the functions of $GABA_AR$ subtypes had been limited by the compensatory up- or down- regulations of other $GABA_AR$ subunits (α1 knock-out, Sur et al., 2001, Vicini et al., 2001), lethal phenotype (γ2 knock-out, Gunther et al, 1995) or absence of detectable behavioral effects (β2 knock-out, Sur et al., 2001). The presence of a histidine residue at a conserved location in the genes encoding α1 , α2 , α3 or α5 subunit has been shown to be critical for diazepam sensitivity of the corresponding $GABA_AR$ subtypes. α4 and α6 $GABA_A$ R have an arginine residue at this site, which renders them insensitive to diazepam and other classical BDZ (Wieland et al., 1992). Recombinant

receptor studies done by replacing the histidine residue with an arginine residue confirmed the loss of potentiating effect of diazepam without affecting the receptor sensitivity to GABA (Benson et al., 1998; Dixon et al., 2011).

This knowledge helped to generate $GABA_AR$ α subunit point-mutated ("knock-in") mice, which were rendered insensitive to diazepam by replacing the histidine residue with arginine (Rudolph et al., 2001). Knock-in mice carrying these point-mutations (α1(H101R), α2(H101R), α3(H126R), α5(H105R)) enabled the characterization of defined $GABA_AR$ subtypes mediating the sedative, anxiolytic, anticonvulsant and myorelaxant actions of diazepam (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2001; Crestani et al., 2002), as well as its antinociceptive effects in models of chronic pain. Thus, α1 $GABA_AR$ mediate sedative, anticonvulsant, anterograde amnesic effects of diazepam (Rudolph et al., 1999; McKernan et al., 2000; Crestani et al., 2002; Savic et al., 2006); whereas α2 $GABA_AR$ selectively mediate anxiolytic and alcohol potentiating effects of diazepam, as well as parts of its antihyperalgesic action. α3 and α5 $GABA_AR$ contribute moderate-low modulations in nociception (Löw et al., 2000; Täuber et al., 2003; Knabl et al., 2008). At the same time, the muscle relaxant effect is primarily mediated by α2 and α5 $GABA_AR$ with α3 $GABA_AR$ and shown to be effective at higher doses of diazepam (Crestani et al., 2001; Crestani et al., 2002). Sedative tolerance to diazepam administration depends on α5 $GABA_AR$ (van Rijnsoever et al., 2004). More importantly, the knock-in mouse lines did not show a decrease in the mutated subunit or apparent compensatory regulations of other $GABA_AR$ subunits (Möhler et al., 2002) except in α5 (H105R) mice with a reduced expression of α5 subunit in the medial temporal lobe (Crestani et al., 2002; Prut et al., 2010).

The availability of *cre/loxP* system that provides site-specific gene recombination (Sauer et al., 1987; Sauer and Henderson, 1988a, 1988b) was also employed for mutating $GABA_AR$ subunits. *Cre/loxP* recombination targets a specific sequence of DNA and its splicing is achieved with *cre* recombinase enzyme (38-kDa). It recognizes a 34-bp DNA segment called "locus of crossing-over of P1" (*loxP*) which was first described in the bacteriophage P1 (Orban et al., 1992; Lakso et al., 1996). This site is unlikely to occur randomly in the mouse genome and is small enough to be "neutral" when integrated into chromosomal DNA. When two *loxP* sites are located on the same DNA molecule, *cre* causes inversion or excision of the intervening DNA segment based on their respective

orientation. Two transgenic mouse lines are required to generate tissue-specific gene deletions. The first mouse line expresses the *cre* recombinase under the control of a tissue-specific promoter. The second carries *loxP* sites flanking around the gene (essential exon) of interest ("floxed gene"). In the offspring from these two lines, the gene (or exons) of interest will be removed selectively from cells expressing *cre* recombinase. This system was employed in creating the transgenic mouse line carrying a specific spinal deletion of $GABA_AR$ α2 subtypes using a mouse line that expresses the *Cre* recombinase under the transcriptional control of the spinally expressed *Hoxb8* gene (Witschi et al., 2010).

1.5.4 Distribution of $GABA_A$ Receptor Subtypes in Rodent Brain

Previous studies have revealed the distinct distribution pattern of $GABA_AR$ subtypes in the murine brain (Fritschy et al., 1992; Fritschy and Mohler, 1995; Pirker et al., 2000; Möhler et al., 2002). Diazepam-sensitive $GABA_AR$ are the most abundant in the brain altogether constituting 90% of the total GABAR. α1 $GABA_AR$ alone represent 60% out of the total $GABA_AR$ (Benke et al., 1991). The α1 subunit is abundantly present in the cerebral cortex, hippocampus, thalamus, cerebellum and olfactory bulb at both synaptic and extrasynaptic sites, whereas it is absent in a few brain regions including the granule cell layer of olfactory bulb and the reticular nucleus of the thalamus. The α2 subtypes contribute to 15-20% $GABA_AR$, which are present at synaptic locations of cerebral cortex, hippocampus and striatum. The α3 subtypes are located mainly in synapses of cerebral cortex, thalamic reticular nucleus, as well as in monaminergic cells of brain stem and cholinergic neurons, accounting for up to 10-15% of the total $GABA_AR$. The α5 subunit is less abundant in the rat brain (< 5%) except in hippocampus. A rough complementary pattern of distribution is observed with α1 versus α2 and α3 $GABA_AR$. Diazepam-insensitive $GABA_AR$ represent 6% of the total $GABA_AR$. The α6 subunit is present exclusively in cerebellar granule cells, both at synaptic and extrasynaptic locations whereas the α4 subunit has been found only extrasynaptically.

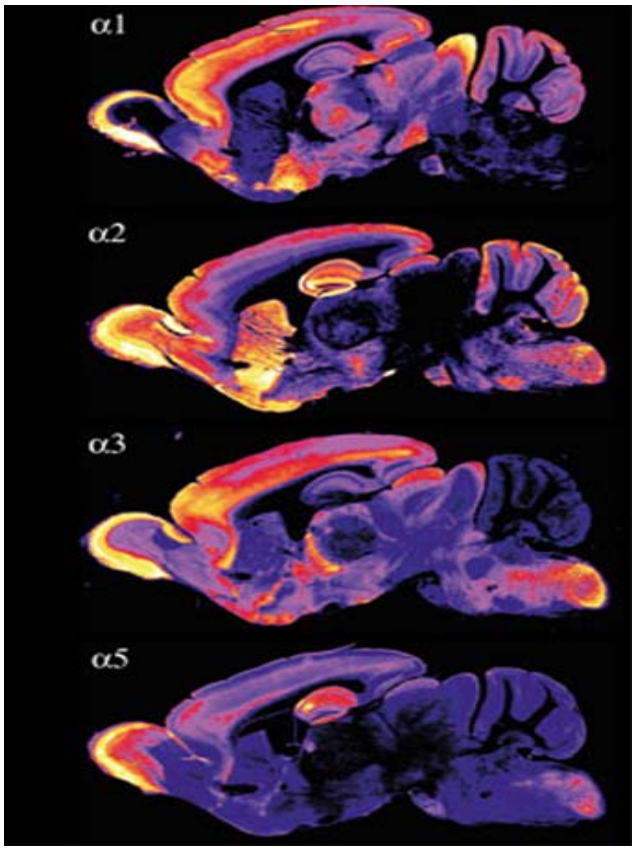


Fig. 6. Distinct α subunit distribution pattern, reflecting the distribution of diazepam-sensitive $GABA_A$ receptors in the murine brain.
The $GABA_A$ receptor α1 subunit is abundant in cerebral cortex, cerebellar cortex, thalamus and pallidum. The α2 subunit is localized to striatum, hippocampus, amygdala and hypothalamus. The α3 subunit is expressed in the monaminergic and serotonergic neurons of the brain stem and the cholinergic neurons of the basal forebrain. The α5 subunit is found predominantly in the hippocampus and olfactory bulb. Adapted from Mohler et al. (2002).

1.5.5 Distribution of $GABA_A$ Receptor Subtypes in Rodent Spinal Cord

Compared to brain, distribution of $GABA_AR$ subtypes in spinal dorsal horn is relatively less well explored. In rat spinal cord, *in situ* hybridization studies detected strong signals for $GABA_AR$ α2, α3, β3 and γ2 subunit mRNA, weak expression for α1, α5, β1, β2, γ1 and γ3, but could not detect α6 and δ subunits (Persohn et al., 1991; Wisden et al., 1991; Ma et al., 1993). Immunohistochemical analysis has shown that α1, α2 and α5 subunit show restricted lamina-specific distribution whereas the α3 subunit is more widely expressed (Bohlhalter et al., 1996). Previous work in our laboratory showed that α2 and α3 subunit correspond to the most abundant diazepam-sensitive $GABA_AR$ subtypes in the mouse spinal dorsal horn and that intrinsic dorsal horn neurons express mainly the α2 and α3 subunits (Knabl et al., 2008).

1.5.6 Interaction of Gephyrin with $GABA_A$ Receptor Subtypes

Gephyrin was first discovered as a glycine receptor subunit (Pfeiffer et al., 1982) and was later recognized as a scaffolding protein that interacts with polymerized tubulin and anchors glycine receptors in the membrane (Kirsch et al., 1991). In 1995, gephyrin was

discovered to be present in GABAergic synapses of retina and later in brain (Sassoè Pognetto et al., 1995; Sassoè Pognetto et al., 2000). Antisense treatment against gephyrin in hippocampal cultures resulted in a significant decrease of postsynaptic α2 subunit containing $GABA_AR$ clusters, indicating that gephyrin is involved in $GABA_AR$ clustering (Essrich et al., 1998). Furthermore, in hippocampal cultures, transfected with GFP-tagged $GABA_AR$, the receptors were found to be more mobile in the plasma membrane when gephyrin is silenced by RNA interference, suggesting that $GABA_AR$ clusters are stabilized via gephyrin (Jacob et al., 2005). Besides this, $GABA_AR$ α2 subtype accumulation in inhibitory postsynaptic sites directly correlates with their ability to bind to gephyrin (Tretter et al., 2008). Thus, in analogy with PSD-95 in excitatory synapses, gephyrin is thought to be a scaffolding protein holding glycine/$GABA_AR$ at inhibitory postsynaptic sites.

1.5.7 Role of $GABA_A$ Receptor Subtypes in Nociception

Several lines of evidence indicate that $GABA_AR$ present in the spinal dorsal horn are the major regulators of the normal and pathological form of pain, notably in comparison to supraspinal $GABA_AR$ (Jasmin et al., 2003; Knabl et al., 2008; Munn et al., 2009; Munro et al., 2011). In principle, $GABA_AR$ modulate spinal nociceptive processing via at least two sites. Postsynaptic $GABA_AR$ directly reduce the excitability of central dorsal horn neurons, while $GABA_AR$ located on the spinal nociceptive terminals mediate presynaptic inhibition. Morphological data employing immunofluorescence and *in situ* hybridization support the possibility that α2 $GABA_AR$ mediate most of the analgesic effects of BDZ applied intrathecally (Knabl et al., 2008) and are the major $GABA_AR$ isoform in DRG neurons (Ma et al., 1993). Besides, co-staining experiments with antibody against substance P revealed that α2, but not α1, α3 or α5 $GABA_AR$ were extensively colocalized with peptidergic nociceptive terminals in lamina II. $GABA_AR$ containing α3 subunit were also identified as a component of the spinal pain control and were found colocalized with NK1-receptor-positive lamina I neurons (Knabl et al., 2008). Negative allosteric modulation of α5 $GABA_AR$ using systemic α5IA-II resulted in analgesia, suggesting a potential role of these receptors in activation of spinally-mediated antihyperalgesic effects (Munro et al., 2011). So far, the neuroanatomical locations of $GABA_AR$ α1, α4, and α5 subunit containing neurons and afferent terminals in the spinal dorsal horn are rather unclear.

The inhibitory interneurons in the spinal dorsal horn serve important functions in the central processing of sensory information. The available literature points towards a prominent role of $GABA_AR$ α2 subunit containing subtypes in the regulations of antihyperalgesia in the dorsal horn. Thus, the projects detailed in this dissertation aim to explore the contribution of nociceptive versus non-nociceptive terminal residing α2 $GABA_AR$. Besides, the contribution of spinally versus supraspinally located α2 $GABA_AR$ towards antihyperalgesia in inflammatory and neuropathic models is also investigated. Ultimately, this dissertation contributes to the knowledge pool that might result in the development of novel pharmacological approaches to pain treatment.

AIMS

2 Aims

Despite numerous advances in our understanding of the functional diversity of $GABA_AR$ subtypes and their profound influence on nociception, their exact cellular distribution pattern in the spinal dorsal horn is largely unknown. Several lines of evidence suggest that pathological pain of inflammatory or neuropathic origin converge onto a loss of synaptic inhibition in the spinal cord, which should be reversible through the potentiation of GABAergic neurotransmission. In line with this hypothesis, BDZ, which facilitate the action of GABA at $GABA_AR$, exert clear antihyperalgesic actions after local spinal application, both in animal models (Clavier et al., 1992; Sumida et al., 1995) and in patients (Tucker et al., 2004). This thesis combines pharmacological, behavioral, electrophysiological, and morphological experiments in wild type and genetically modified mice to unravel the localization of the $GABA_AR$ α subtypes in the mouse spinal cord and to identify the specific sites of action of α2 $GABA_AR$-mediated antihyperalgesia.

2.1 Contribution of Presynaptic α2 $GABA_A$ Receptors Located on the Central Terminals of Primary Nociceptive Afferents to the Spinal Nociceptive Processing.

At the spinal cord level, $GABA_AR$ can in principle modulate nociceptive processing via at least two sites. Postsynaptically located $GABA_AR$ directly reduce the excitability of central dorsal horn neurons, while $GABA_AR$ located presynaptically on the spinal terminals of primary afferent nociceptors cause presynaptic inhibition. Morphological data employing immunofluorescence and *in-situ* hybridization have suggested that α2 $GABA_AR$, which mediate most of the analgesic effects of spinal BDZ (Knabl et al., 2008), are the major if not the only $GABA_AR$ isoform in dorsal root ganglion neurons (Ma et al., 1993). In addition, co-staining experiments with antibodies against substance P revealed that α2 $GABA_AR$ were extensively colocalized with peptidergic primary afferent nociceptive terminals in lamina II. α3 $GABA_AR$ were also identified as a critical component of the spinal pain control and were present in NK1-receptor-positive lamina I neurons (Knabl et al., 2008). To address the contribution of presynaptic α2 $GABA_AR$ to the analgesic effects of spinal BDZ, mice were generated carrying targeted deletions or

mutations of the $GABA_AR$ α2 subunit in primary nociceptive neurons. Behavioral, morphological and electrophysiological experiments were performed to analyze the phenotype of $GABA_AR$ mutant mice in different models of inflammatory and neuropathic pain in absence of treatment and after intrathecal injection of diazepam.

2.2 Contribution of Spinal $GABA_A$ Receptor α2 Subtype to the Nociceptive Regulation.

This project aims to distinguish the contribution of spinal versus supraspinal α2 $GABA_AR$ to antihyperalgesia induced by BDZ-site ligands using a mouse line expressing the Cre recombinase under the transcriptional control of the homeobox gene *Hoxb8* (Witschi et al., 2010), together with a floxed *Gabra2* gene. Within the neuraxis, *Hoxb8*-Cre expression is restricted to spinal cord neurons and glial cells, as well as dorsal root ganglion neurons, allowing selective excision of a floxed gene in these cells. Since *Hoxb8* is expressed only up to the mid cervical segments during development, *Gabra2* is not affected in the supraspinal cells. Behavioral, biochemical, morphological and electrophysiological experiments were performed to analyze the phenotype of these mutant mice in a neuropathic pain model in the absence of treatment and after systemic injection of HZ166, a less sedating BDZ-binding site ligand with preferential affinity for α2 and α3 $GABA_AR$.

2.3 Molecular Organization of $GABA_A$ Receptor Subtypes in the Spinal Dorsal Horn.

This study aims to analyze immunohistochemically the molecular organization of $GABA_AR$ in identified intrinsic dorsal horn neurons and primary afferents in layers I-III of the dorsal horn, using high resolution immunofluorescence staining combined with specific neuronal and axonal markers (Schneider Gasser et al., 2006).

EXPERIMENTAL SECTION

3 Results

3.1 Presynaptic α2 $GABA_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control

Robert Witschi[1,2,*], Pradeep Punnakkal[1,*], Jolly Paul[1,*], Jean-Sébastien Walczak[3], Fernando Cervero[3], Jean-Marc Fritschy[1], Rohini Kuner[4], Ruth Keist[1], Uwe Rudolph[5#], Hanns Ulrich Zeilhofer[1,2#]

[1]Institute of Pharmacology and Toxicology, University of Zurich, CH-8057 Zurich, Switzerland

[2]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) CH-8093 Zurich, Switzerland

[3]Department of Anesthesia, McGill University, Montreal, Québec H3G 1Y6, Canada

[4]Institute of Pharmacology, University of Heidelberg, D-69120 Heidelberg, Germany

[5]Laboratory of Genetic Neuropharmacology, McLean Hospital and Department of Psychiatry, Harvard Medical School, Belmont, Massachusetts 02478

* and # indicate equal contribution.

Published in The Journal of Neuroscience (2011) 31:8134–8142.

Author contributions: R.W., P.P., J.-S.W., F.C., J.-M.F., R. Kuner, and H.U.Z. designed research; R.W., P.P., J.P., J.-S.W., J.-M.F., R. Keist, and U.R. performed research; R. Keist and U.R. contributed unpublished reagents/analytic tools; R.W., P.P., JP., J.-S.W., F.C., J.-M.F., and H.U.Z. analyzed data; H.U.Z. wrote the paper.

Abstract

Spinal dorsal horn $GABA_A$ receptors are found both postsynaptically on central neurons and presynaptically on axons and/or terminals of primary sensory neurons, where they mediate primary afferent depolarization (PAD) and presynaptic inhibition. Both phenomena have been studied extensively on a cellular level, but their role in sensory processing *in vivo* has remained elusive, due to inherent difficulties to selectively interfere with presynaptic receptors. Here, we address the contribution of a major subpopulation of $GABA_AR$ (those containing the α2 subunit) to spinal pain control in mice lacking α2 $GABA_AR$ specifically in primary nociceptors (*sns*-$\alpha2^{-/-}$ mice). *sns*-$\alpha2^{-/-}$ mice exhibited $GABA_AR$ currents and dorsal root potentials of normal amplitude *in vitro*, and normal response thresholds to thermal and mechanical stimulation *in vivo*, and developed normal inflammatory and neuropathic pain sensitization. However, the positive allosteric $GABA_AR$ modulator diazepam (DZP) had almost completely lost its potentiating effect on PAD and presynaptic inhibition *in vitro*, and a major part of its spinal antihyperalgesic action against inflammatory hyperalgesia *in vivo*. Our results thus show that part of the antihyperalgesic action of spinally applied DZP occurs through facilitated activation of $GABA_AR$ residing on primary nociceptors.

Introduction

$GABA_A$ receptors mediate fast synaptic inhibition throughout the adult mammalian CNS. They are also densely expressed in the spinal dorsal horn (Bohlhalter et al., 1996) where they control the propagation of nociceptive signals (Roberts et al., 1986; Ishikawa et al., 2000). Diminished GABAergic and glycinergic inhibition at this site is a major factor in chronic pain syndromes (for a review see (Zeilhofer, 2008)). Conversely, hyperalgesia originating from inflammatory and neuropathic diseases can be reversed by local spinal or systemic administration of benzodiazepines (BDZ) such as diazepam (DZP) (Knabl et al., 2008) and midazolam (Kontinen and Dickenson, 2000), which enhance $GABA_AR$ activation. $GABA_AR$ are heteropentameric ligand-gated ion channels, most of which are composed of α, β and γ subunits (Olsen and Sieghart, 2008). BDZ-sensitive subtypes contain one γ2 subunit, which together with an α1, α2, α3, or α5 subunit forms the BDZ binding site (Pritchett et al., 1989; Wieland et al., 1992). For each of these subunits, point mutated mice have been generated which carry a histidine to arginine (H/R) substitution that destroys the DZP-sensitivity of the mutated α subunit without changing its responses to GABA (Mohler et al., 2002). Using these mice it has become possible to attribute to α2 $GABA_AR$ most of the antihyperalgesic effect of spinal DZP (Knabl et al., 2008).

In the spinal cord α2 $GABA_AR$ are densely expressed in the superficial layers of the dorsal horn, the main termination area of primary nociceptors (Bohlhalter et al., 1996). At this site, α2 $GABA_AR$ are found not only postsynaptically on central neurons, where they cause classical hyperpolarization, but most likely also presynaptically on the terminals of primary sensory neurons (discussed in Persohn et al., 1991, and Bohlhalter et al., 1996). These terminals are depolarized by $GABA_AR$ (Labrakakis et al., 2003), because their intracellular chloride concentration is kept above electrochemical equilibrium by the chloride importer NKCC1 (Alvarez-Leefmans, 2009). This primary afferent depolarization (PAD) causes presynaptic inhibition, i.e. a reduction in synaptic glutamate release, possibly through inactivation of presynaptic sodium channels and/or through activation of a shunting conductance, both of which can result in inhibition of action potential propagation into presynaptic terminals (Kullmann et al., 2005). Both processes will result in reduction of nociceptive input to the spinal cord. However, if

PAD becomes sufficiently strong to trigger action potentials, it may also elicit so called dorsal root reflexes, and exaggerate pain and neurogenic inflammation (Willis, 1999).

The contribution of PAD to the processing of nociceptive signals and to the antihyperalgesic effect $GABA_AR$ modulators is unknown, mainly due to the lack of suitable tools for the specific targeting of presynaptic $GABA_AR$. Here, we used a genetic approach and investigated conditional nociceptor-specific α2 $GABA_AR$-deficient and point mutated mice in morphological, electrophysiological and behavioral experiments. Deletion of α2 $GABA_AR$ in nociceptive primary afferents reduced DZP sensitivity of GABAergic membrane currents in nociceptive dorsal root ganglion (DRG) neurons and $GABA_AR$-mediated presynaptic inhibition, and led to a reduction in the antihyperalgesic effect of spinal DZP.

Materials and Methods

Mice

To generate a floxed *Gabra2* allele, a 6.3 kb *Pst*I-*Nco*I genomic fragment containing exons 5 (221 bp) and 6 (83 bp) together with 2 *Sph*I sites was isolated. The 1 kb *Sph*I-*Sph*I fragment was removed from the 6.3 kb *Pst*I-*Nco*I fragment and replaced by an oligo hybrid containing a *lox*P site with adjacent *Kpn*I and *Sal*I sites, recreating a single *Sph*I site, into which the 1 kb *Sph*I-*Sph*I fragment containing exon 5 was reinserted. A neomycine resistance cassette (FRT-Pol2-neo-bpA-FRT-*lox*P) was then subcloned into the *Sal*I site. The vector was linearized at the 5′ end of the genomic homology at a *Not*I site and electroporated into embryonic stem (ES) cells (C57BL/6N, Eurogentec). Clones harboring a single targeting event ("targeted allele" in fig. 1A) were injected into blastocysts (Polygene, Ruemlang, Switzerland). The neomycine resistance cassette was bred out using ACTFlpe mice (Jackson Laboratories, Bar Harbor, ME, USA) to obtain the floxed allele (Gabra2$^{tm2.1Uru}$). Floxed mice were crossed with *EIIa*-cre mice (Jackson Laboratories) to obtain *Gabra2* global knock-out mice (allele designated Gabra2$^{tm2.2Uru}$). Nociceptor specific *sns*-α2$^{-/-}$ mice and *sns*-α2$^{-/R}$ point mutated mice were generated from *sns*-cre transgenic mice (Agarwal et al., 2004) crossed with α2$^{fl/fl}$ and or α2$^{R/R}$ mice (Low et al., 2000) (for the designations of the different genotypes see Tab. 1). All mice were maintained on a C57BL/6 background.

mRNA quantification

4 - 6 lumbar DRGs, lumbar spinal cords, and cerebral cortices were rapidly removed from euthanized adult *sns*-α2$^{-/-}$ mice and α2$^{fl/fl}$ littermates, as well as from global α2$^{-/-}$ mice. mRNA expression of $GABA_AR$ α subunits was quantified with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using β-actin as reference gene (for Taqman® assays see Tab. 2).

Genotype			***Gabra2* phenotype**		
Gabra2 allele 1	*Gabra2* allele 2	*sns*-cre transgene	Primary nociceptors	All other cell types	Designation (short)
Fl	fl	-	wt / wt	wt / wt	$\alpha2^{fl/fl}$_*sns*-cre^{tg-} ($\alpha2^{fl/fl}$)
Fl	fl	+	- / -	wt / wt	$\alpha2^{fl/fl}$_*sns*-cre^{tg+} (sns-$\alpha2^{/-}$)
Fl	H	-	wt / wt	wt / wt	$\alpha2^{fl/H}$_*sns*-cre^{tg-} ($\alpha2^{fl/H}$)
Fl	H	+	- / wt	wt / wt	$\alpha2^{fl/H}$_*sns*-cre^{tg+} (sns-$\alpha2^{/H}$)
Fl	R	-	wt / R	wt / R	$\alpha2^{fl/R}$_*sns*-cre^{tg-} ($\alpha2^{fl/R}$)
Fl	R	+	- / R	wt / R	$\alpha2^{fl/R}$_*sns*-cre^{tg+} (*sns*-$\alpha2^{/R}$)
R	R	-	R / R	R / R	($\alpha2^{R/R}$)
-	-	-	- / -	- / -	($\alpha2^{/-}$)

Tab. 1. Genotypes and cell type-specific phenotypes of the mouse lines analyzed. Phenotypically, floxed alleles are regarded as wild-type in the absence of cre.

assay ID	**context sequence (including the probe sequence)**	**Gene**
Mm00607939_s1	CTGTTACTGAGCTGCGTTTTACACC	*Actb*
Mm00439046_m1	TTCCAGAAAAGCCAAAGAAAGTAAA	*gabra1*
Mm00433435_m1*	TATATACCATGAGGCTTACAGTCCA	*gabra2*
Mm00433440_m1	AGTGACTGTGACACTCGATCTCACA	*gabra3*
Mm00802631_m1	GAAACATCCCTTCAGAATACACATG	*gabra4*
Mm00621092_m1	ACACCATGCGTCTGACAATCTCTGC	*gabra5*
Mm01227754_m1	CCAGGATTTGGGGGTGCTGTAACAG	*gabra6*
Mm01266203_g1	TCTCAGAGGCAAACATGGAATACAC	*Gabrd*
Mm00489932_m1	CCAGACATGGAATATTCCATTGACA	*Gabre*
Mm01193033_m1	GTAACATGGACTACACAGCCACTAT	*Gabrp*
Mm00445057_m1	AGCAAATGTGCAGGATGGCCTGATT	*Gabrq*
Mm00433499_m1	GCAAGGCAGCCCAATCCTGAGACGA	*gabrr1*
Mm00433507_m1	TCCAAGCCAAGCCATTTGTATAAAA	*gabrr2*
Mm01344096_m1	GTTTCCCTGGGGATCACGACGGTGC	*gabrr3*

Tab. 2. qRT-PCR (Taqman) assays used to quantify $GABA_AR$ α subunit expression. * This assay amplifies a gene segment including the floxed *gabra2* exon 5 and therefore does not yield a PCR product from chromosomes which have undergone *cre*-mediated excision of exon 5.

Morphology

Lumbar spinal cords prepared from 6 - 8 week old *sns*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates were cut into 300 µm thick parasagittal slices, fixed in 4% paraformaldehyde for 10 min and subsequently cut into 14 µm thick sections using a cryostat. Immunofluorescence staining was performed to study the colocalization of $GABA_AR$ α2 and α3 subunits using guinea pig affinity purified antisera (guinea pig affinity purified antisera (Knabl et al., 2008) with markers of primary afferent nociceptive fibers (CGRP and IB4). A polyclonal rabbit antiserum against CGRP (Chemicon, cat. no. AB 15360) and an IB4-Alexa 488 conjugate (Molecular Probes, cat. no. 121411) were used to label spinal axons and terminals of peptidergic and nonpeptidergic nociceptors, respectively. Thick myelinated (non-nociceptive) fiber terminals were labeled with a rabbit antiserum against VGluT1 (Synaptic Systems, Göttingen). High-resolution confocal images were processed and analyzed with Imaris (Bitplane) software. Double-immunofluorescence staining was visualized by confocal microscopy (Zeiss LSM-710 Meta; Jena, Germany) using a 63x Plan-Apochromat objective (NA 1.4). The pinhole was set to 1 Airy unit for each channel and separate color channels were acquired sequentially. The acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. High-resolution confocal images were processed and analyzed with Imaris (Bitplane) with minimal adjustments of contrast and brightness. Images from both channels were overlaid (maximal intensity projection) and background was subtracted, when necessary. A low-pass 'edge preserving' filter was used for images displaying α2 or α3 staining. Colocalization of α2 subunit immunoreactivity with primary afferent terminals was quantified from single confocal sections (1024×1024 pixels) at a magnification of 56 µm / pixel in 8 bit grayscale images, using a threshold segmentation algorithm (minimal intensity, 90 – 130; size 0.08 – 0.8 μm^2). Colocalizations were counted in 6 fields per slide each from a different mouse. 3 mice per genotype were analyzed. Colocalizations were considered to be true, only if (a) the α subunit staining appeared completely inside the primary afferent staining, (b) covered an area > 0.057 μm^2, and (c) the colocalization was visible in the previous and next images of the Z-stack.

Electrophysiology

Whole-cell patch-clamp recordings were made at room temperature from acutely isolated nociceptive DRG neurons and from superficial dorsal horn neurons. DRG neurons were prepared from 3 - 4 week old mice (Knabl et al., 2008). Nociceptive DRG

neurons were identified by the presence of Na^+ currents resistant to TTX (0.3 μM) and exhibiting pronounced reduction in amplitudes during repetitive (5 Hz) depolarizations for 30 ms to 0 mV (Blair and Bean, 2003). Transverse spinal cord slices with short dorsal roots attached were prepared from 2 - 3 week old mice (Ahmadi et al., 2002). Dorsal roots were stimulated electrically (duration ≥ 100 μs; 17 - 70 V) at a frequency of 0.07 Hz to elicit primary afferent-evoked EPSCs. DRP recordings were made from isolated spinal cords of 18 - 27 day old mice at 28.5°C (Martinez-Gomez and Lopez-Garcia, 2005). Dorsal roots S2 or S3 were stimulated and the cranially adjacent root was recorded. Suction electrodes were used for both stimulation and recording.

Behavior

Experiments were done in 7 - 10 week old mice. Care was taken to ensure equal numbers of male and female mice in all experiments. Inflammatory and neuropathic pain induction, thermal and mechanical testing, and intrathecal (i.t.) injections, i.e. injections into the subarachnoid space of the spinal canal, of diazepam and vehicle were done as described previously (Knabl et al., 2008). Capsaicin was dissolved in Tween 80 (10%), ethanol (10%), saline (80%). Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 121/2006 and 34/2007).

Results

Nociceptor-specific α2 $GABA_A$ receptor-deficient mice.

Conditional nociceptor-specific α2 $GABA_AR$ -deficient mice ($\alpha2^{fl/fl}$_*sns*-cre^{tg+}; short *sns*-$\alpha2^{-/-}$ mice) were generated by crossing mice carrying a floxed α2 $GABA_AR$ (*Gabra2*) gene (fig. 1A) to transgenic mice expressing the *cre* recombinase under the transcriptional control of the sensory neuron specific sodium channel (*sns*) gene (Agarwal et al., 2004).

The expression of the other BDZ-sensitive $GABA_AR$ subunits was not significantly changed in DRGs of *sns*-$\alpha2^{-/-}$ mice (fig. 1C). We also analyzed possible changes in the expression of the BDZ-insensitive $GABA_AR$ subunits α4, α6, δ, ε, π, θ, and ρ1-ρ3 (Tab. 3). Transcripts encoding for six of these subunits (α4, δ, ε, θ, ρ1, and ρ3) were reliably detected in DRGs of both $\alpha2^{fl/fl}$ and *sns*-$\alpha2^{-/-}$ mice. mRNA encoding for the α4 subunit was significantly up-regulated in *sns*-$\alpha2^{-/-}$ mice by 44.5 ± 9.5% (mean ± sem). Up-regulations by between 20 and 40% were also found for δ, θ, and ρ1 but these did not reach statistical significance.

No detectable levels of α2 subunit mRNA were found in global $\alpha2^{-/-}$ mice (generated from $\alpha2^{fl/fl}$ mice crossed to *EIIa*-cre mice (Lakso et al., 1996) verifying the specificity of the assay and suggesting that the α2 mRNA remaining in DRGs of *sns*-$\alpha2^{-/-}$ mice derived most likely from non-nociceptive (*sns*-cre negative) DRG neurons.

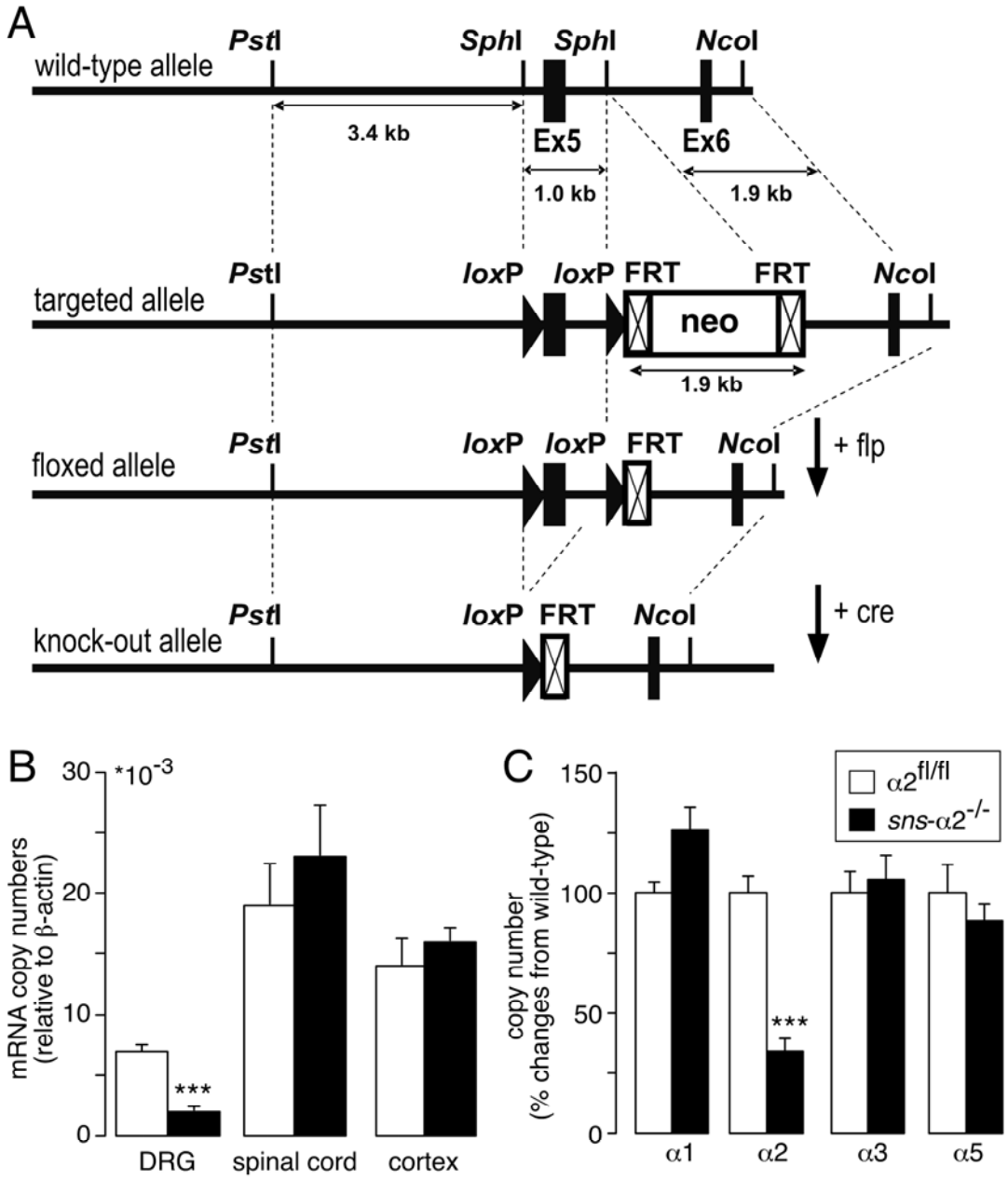


Fig. 1. Generation of $GABA_AR$ $\alpha2^{fl/fl}$ mice and qRT-PCR analyses. (A) Generation of mice carrying a floxed *Gabra2* allele. For details see Materials and Methods. (B) Quantification (mean ± sem) of *Gabra2* transcript numbers (relative to β-actin) in lumbar DRGs, spinal cords and cerebral cortices of *sns*-α2 $^{/-}$ mice (n = 7) and wild-type ($\alpha2^{fl/fl}$) littermates (n = 9) with qRT-PCR. (C) Quantification of *gabra1*, *Gabra2*, *Gabra3* and *Gabra5* gene transcripts (encoding for the BDZ-sensitive subunits α1, α2, α3, and α5) in the DRGs of *sns*-α2 $^{/-}$ mice and wild-type ($\alpha2^{fl/fl}$) littermates. ***, $P \leq 0.001$. Statistical comparisons between wild-type and *sns*-$\alpha2^{-/-}$ were made with unpaired t tests followed by Bonferroni corrections for 3 (B) and 4 (C) independent comparisons.

$GABA_A$ receptor subunit (gene)	**expression relative to β-actin in $\alpha2^{fl/fl}$ mice (mean ± sem)**	**expression ratio (sns-$\alpha2^{-/-}$ / $\alpha2^{fl/fl}$) (mean ± sem)**	***P***
α4 (*Gabra4*)	$7.6 \pm 0.34 \cdot 10^{-5}$	1.45 ± 0.25	0.004
α6 (*Gabra6*)	n.d.		
δ (*Gabrd*)	$2.9 \pm 0.45 \cdot 10^{-4}$	1.41 ± 0.45	n.s.
ε (*Gabre*)	$2.2 \pm 0.21 \cdot 10^{-4}$	1.10 ± 0.26	n.s.
π (*Gabrp*)	n.d.		
θ (*Gabrq*)	$2.5 \pm 0.41 \cdot 10^{-4}$	1.37 ± 0.29	n.s.
ρ1 (*Gabrr1*)	$2.6 \pm 0.39 \cdot 10^{-4}$	1.23 ± 0.14	n.s.
ρ2 (*Gabrr2*)	n.d.		
ρ3 (*Gabrr3*)	$1.8 \pm 0.33 \cdot 10^{-4}$	0.90 ± 0.11	n.s.

Tab. 3. Changes in gene expression in sns-$\alpha2^{-/-}$ mice compared to $\alpha2^{fl/fl}$ mice. Gene expression for both genotypes was first calculated relative to β-actin expression, and then compared between sns-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice. n.d., not detectable. α6 transcripts were not detectable in any of the samples, and transcripts for π and ρ2 were only found in 2 out of 16 samples. *P*, significance calculated by ANOVA followed by Bonferroni correction for 6 independent samples. n.s., not significant after Bonferroni correction. Number of mice, n = 8 and 7, for $\alpha2^{fl/fl}$ and sns-$\alpha2^{-/-}$, respectively.

α2 $GABA_A$ receptors expressed in spinal terminals of primary afferent sensory fibers.

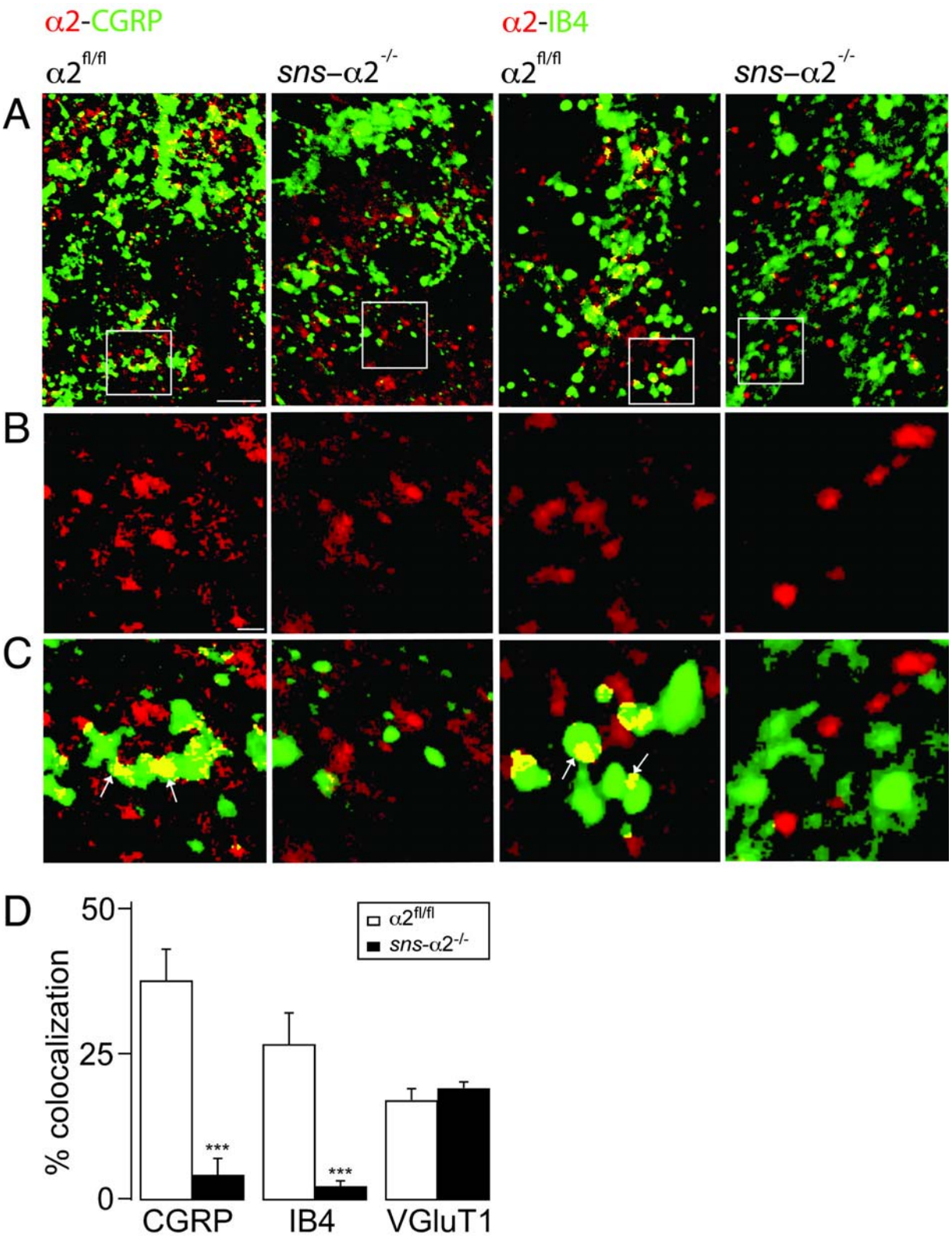
High resolution confocal microscopy was used in parasagittal sections of the lumbar spinal cord to quantify the expression of α2 $GABA_AR$ in the three major subpopulations of primary afferent fibers. Peptidergic and nonpeptidergic nociceptive fiber axons and terminals were labeled with antiserum against calcitonin gene related peptide (CGRP) and with a fluorescent isolectin B4 (IB4) conjugate, respectively, while non-nociceptive fiber terminals were labeled with an antiserum against the vesicular glutamate transporter 1 (VGluT1), which is in the dorsal horn selectively expressed by thick myelinated (non-nociceptive) primary afferent fiber terminals (Todd et al., 2003). All sections were counterstained with an antiserum against the $GABA_AR$ α2 subunit (fig. 2A,B). In the major termination area of nociceptive fibers (laminae I and II of the spinal

dorsal horn), about one third and one fourth of CGRP and IB4 positive structures stained also positive for α2 $GABA_AR$ in wild-type ($\alpha2^{fl/fl}$) mice. These colocalizations were virtually absent in *sns*-$\alpha2^{-/-}$mice (fig. 2C,D). As expected, VGluT1 positive structures were mainly located in the deep dorsal horn (lamina III and deeper). They also showed a considerable but lower degree of colocalization with α2 $GABA_AR$, which was unchanged in *sns*-$\alpha2^{-/-}$ mice. We also found a significant expression of α3 $GABA_AR$ in all three types of primary afferent fibers (52 ± 12%, 41 ± 16%, 27 ± 4% (mean ± SD) with CGRP, IB4 and VGluT1, respectively). The distribution of α3 $GABA_AR$ was not altered in *sns*-$\alpha2^{-/-}$ mice.

Electrophysiological analysis of *sns*-$\alpha2^{-/-}$ mice.

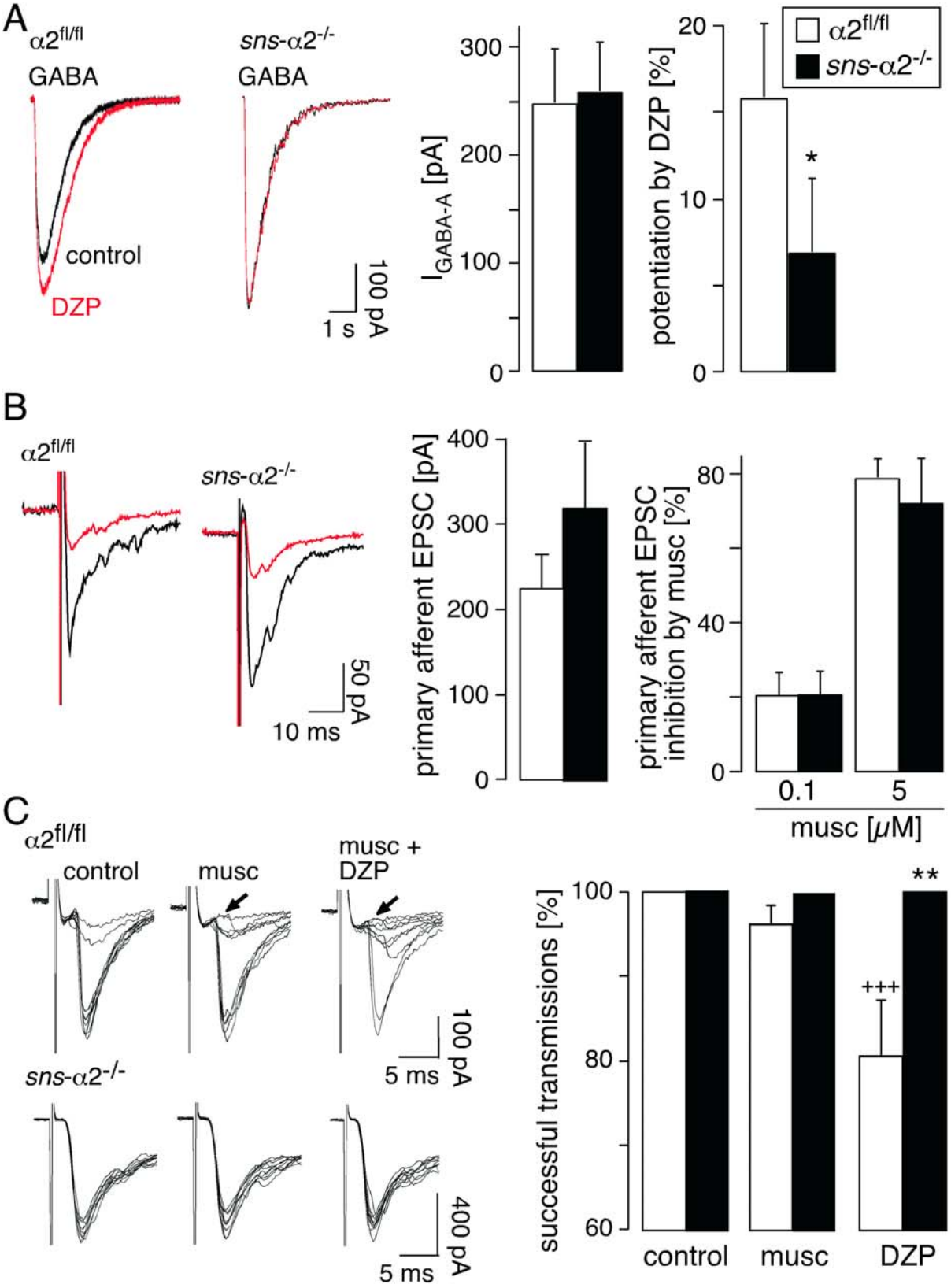
To analyze functional consequences of *sns*-α2 gene deletion at the cellular level, we first made whole-cell recordings from acutely isolated nociceptive DRG neurons identified by the presence of tetrodotoxin (TTX)-resistant Na^+ currents with pronounced use-dependent inactivation upon repetitive stimulation (Pearce and Duchen, 1994; Arbuckle and Docherty, 1995; Blair and Bean, 2003). Amplitudes of GABAergic membrane currents evoked by exogenous application of muscimol remained unchanged in *sns*-$\alpha2^{-/-}$ mice, but their facilitation by DZP (1 μM) was significantly reduced (fig. 3A).

Fig. 2. α2 $GABA_AR$ in the spinal dorsal horn. (A) Colocalization of α2 $GABA_AR$ (red) with peptidergic (CGRP positive, lamina II outer) and nonpeptidergic (IB4 positive, lamina II inner) axons and terminals (green) in parasagittal sections of lumbar spinal cord of adult wild-type ($\alpha2^{fl/fl}$) and *sns*-$\alpha2^{-/-}$mice. (B, C) Higher magnification of the areas indicated in (A) showing the α2 subunit immunoreactivity alone (B) or superimposed with colocalized pixels (yellow, C). Arrows in C point to the terminals containing the α2 $GABA_AR$. (B) α2 $GABA_AR$ immunoreactivity. (C) Colocalization (indicated by arrows). (D) Statistical analysis. Percent colocalization (mean ± sd) of CGRP (lamina IIo), IB4 (lamina IIi) and VGluT1 (lamina III) positive axons and terminals with α2 $GABA_AR$. Colocalizations (for criteria see methods) were counted in 6 fields per slide each from a different mouse. 3 mice per genotype were analyzed. ANOVA followed by Bonferroni post hoc test $F(5,12) = 47.0$; ***, $P \leq 0.001$. Scale bars 5 μm (A) and 0.5 μm (B,C).


α2-CGRP
α2-IB4
α2fl/fl
sns–α2-/-
α2fl/fl
sns–α2-/-
A
B
C
D
50
25
0
% colocalization
α2fl/fl
sns-α2-/-
***
***
CGRP
IB4
VGluT1

We next analyzed the modulation of primary afferent-evoked synaptic transmission by presynaptic $GABA_AR$ in transverse spinal cord slices. AMPA receptor-mediated excitatory postsynaptic currents (EPSC) were evoked by electrical stimulation of attached dorsal rootlets and recorded from visually identified superficial (laminae I/II) dorsal horn neurons. Electrical stimulation thresholds of AMPA-EPSCs were virtually identical in wild-type and *sns*-$\alpha2^{-/-}$ mice (32.4 ± 2.9 V [n = 17] and 34.1 ± 2.8 V [n = 10], means ± sem). In the absence of muscimol or DZP, the vast majority of AMPA-EPSCs were reliably triggered by dorsal root stimulation and occurred with constant latencies. They therefore most likely represented monosynaptic events. After a stable AMPA-EPSC was established slices were superfused with different concentrations of muscimol to activate $GABA_AR$. To avoid confounding effects arising from activation of postsynaptic $GABA_AR$, we replaced in the intracellular recording solution Cl^- with F^- (Turecek and Trussell, 2001), which does not permeate $GABA_AR$ channels (Bormann et al., 1987). AMPA-EPSC amplitudes were not significantly different between *sns*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates, and were similarly decreased with the $GABA_AR$ agonist muscimol in both genotypes (fig. 3B). However, when DZP (1 μM) was applied in addition to a low concentration (0.1 μM) of muscimol, the rate of successful transmissions (i.e. of presynaptic stimulations eliciting EPSCs) dropped significantly in $\alpha2^{fl/fl}$ mice as expected for a presynaptic site of action. This increased inhibition was not observed in *sns*-$\alpha2^{-/-}$ mice (fig. 3C).

Fig. 3. GABAergic membrane currents and primary afferent-evoked synaptic transmission in wild-type ($\alpha2^{fl/fl}$) and *sns*-$\alpha2^{-/-}$ mice. (A) GABAergic membrane currents recorded from nociceptive DRG neurons. Left: individual current traces evoked through puffer application of GABA (1 mM) to the soma of the recorded DRG neuron in $\alpha2^{fl/fl}$ and *sns*-$\alpha2^{-/-}$ mice in the absence (black) or presence (red) of DZP (1 μM). Right: statistical analysis (mean ± sem). n = 26 ($\alpha2^{fl/fl}$) and 14 (*sns*-$\alpha2^{-/-}$). *, $P < 0.05$ (unpaired t test). (B, C) Primary afferent-evoked EPSCs recorded from lamina I/II neurons in transverse spinal cord slices. (B) left: current traces under control conditions (black) and in the presence of muscimol (musc, 5 μM, red). Right: statistical analysis (mean ± sem). EPSC amplitudes: unpaired t test, n = 19 ($\alpha2^{fl/fl}$), n = 18 (*sns*-$\alpha2^{-/-}$); inhibition by muscimol, n = 6 - 17. (C) Analyses of synaptic failure rates. Left: superposition of 10 consecutive primary afferent-evoked EPSCs under control conditions, in the presence of muscimol (0.1 μM), and in the additional presence of DZP (1 μM). Right: statistics (mean ± sem). n = 17 ($\alpha2^{fl/fl}$) and 10 (*sns*-$\alpha2^{-/-}$). ANOVA (genotype * treatment); F (3,81) = 3.96; *, P = 0.03; **, $P < 0.01$ significant against $\alpha2^{fl/fl}$; +++, $P < 0.001$ significant against control.

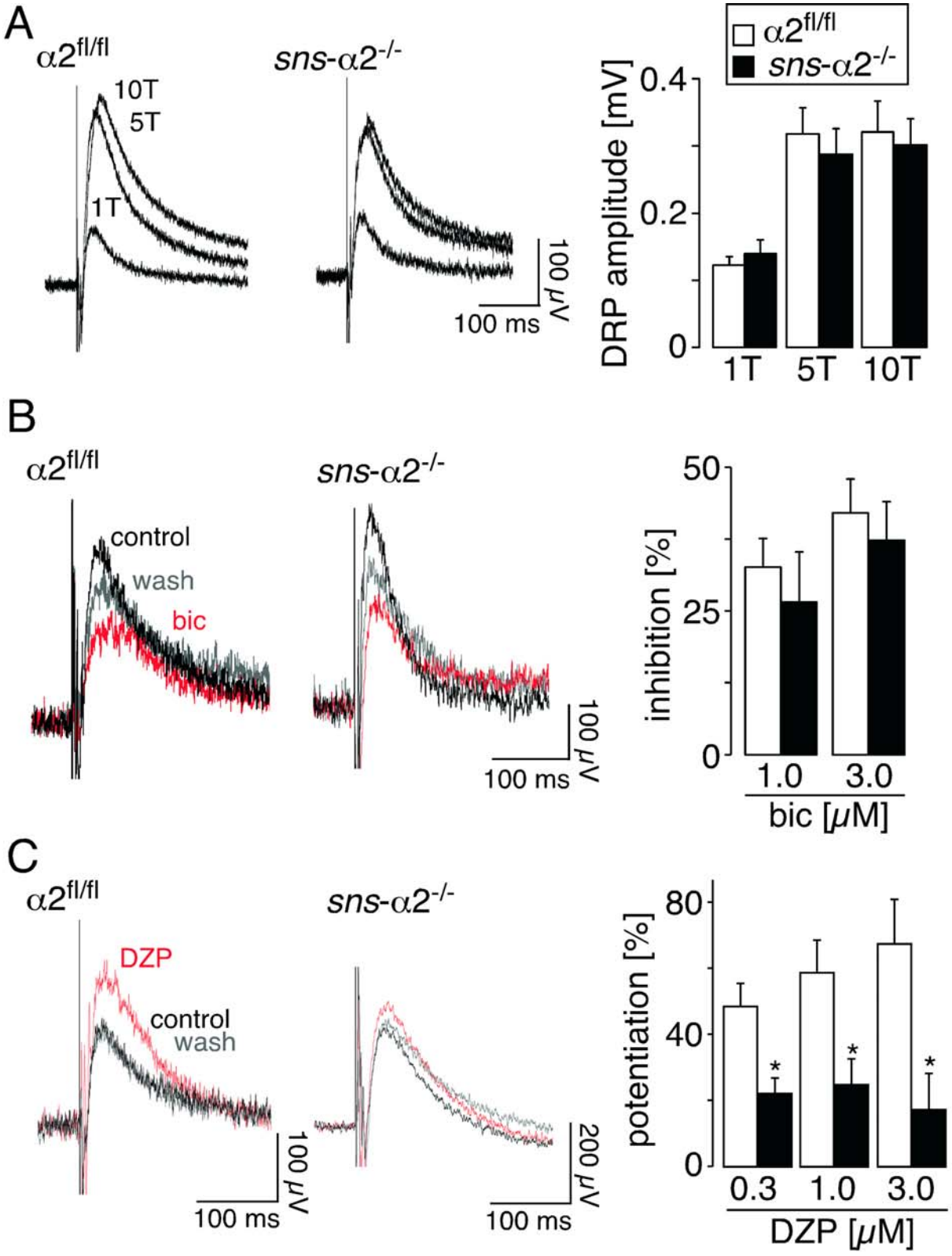

A
α2fl/fl
GABA
control
DZP
sns-α2-/-
GABA
100 pA
1 s
I GABA-A [pA]
300
200
100
0
potentiation by DZP [%]
20
10
0
α2fl/fl
sns-α2-/-
*
B
α2fl/fl
sns-α2-/-
50 pA
10 ms
primary afferent EPSC [pA]
400
300
200
100
0
primary afferent EPSC inhibition by musc [%]
80
40
0
0.1
5
musc [μM]
C
α2fl/fl
control
musc
musc + DZP
100 pA
5 ms
sns-α2-/-
400 pA
5 ms
successful transmissions [%]
100
80
60
**
+++
control
musc
DZP

The functioning of $GABA_AR$ on the presynaptic terminals of primary nociceptors was also assessed through the analysis of dorsal root potentials (DRPs). These are local field potentials generated by GABAergic interneurons and occurring in one dorsal root after electrical stimulation of another dorsal root in a neighboring segment. We compared DRPs of *sns*-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice in terms of amplitude, sensitivity to the $GABA_AR$ blocker bicuculline, and DZP sensitivity. DRPs of *sns*-$\alpha2^{-/-}$ mice were of similar size and similarly sensitive to bicuculline (1 and 3 µM), but their potentiation by DZP was strongly reduced (0.3 – 3 µM) (fig. 4).

Acute nociception and inflammatory and neuropathic hyperalgesia in *sns*-$\alpha2^{-/-}$ mice.

Before analyzing conditional α2 $GABA_AR$ mutant mice, we verified that the presence of the *sns*-cre transgene alone did not affect the development of hyperalgesia or the responsiveness of mice to diazepam. *sns*-cre mice with no mutations in the *Gabra2* gene developed normal hyperalgesia and responded normally to spinal DZP (Tab. 4). We then continue with the analysis of *sns*-$\alpha2^{-/-}$ mice. These mice responded normally to acute noxious heat and to mechanical stimulation with von Frey filaments and exhibited normal nociceptive responses (flinches) after chemical activation of nociceptors through subcutaneous capsaicin injection into one hind paw (Tab. 5).

Fig. 4. Dorsal root potentials. (A) Left, average traces of DRPs recorded at threshold stimulation (1T) and at 5-fold (5T) and 10-fold (10T) higher stimulation intensities in wild-type ($\alpha2^{fl/fl}$) and *sns*-$\alpha2^{-/-}$ mice. Right, statistical analysis (mean ± sem). n = 18 ($\alpha2^{fl/fl}$) and 14 (*sns*-$\alpha2^{-/-}$). (B) Same as (A), but inhibition by bicuculline (bic, 1.0 µM, red) of DRPs elicited at 5T. n = 9 ($\alpha2^{fl/fl}$) and 7 (*sns*-$\alpha2^{-/-}$). (C) Same as (B) but potentiation by DZP (1 µM, red). n = 9 ($\alpha2^{fl/fl}$) and 5 (*sns*-$\alpha2^{-/-}$). *, $P < 0.05$ (unpaired t test) significant against $\alpha2^{fl/fl}$.


A
α2fl/fl
sns-α2-/-
10T
5T
1T
100 μV
100 ms
α2fl/fl
sns-α2-/-
DRP amplitude [mV]
0.4
0.2
0
1T
5T
10T
B
α2fl/fl
sns-α2-/-
control
wash
bic
100 μV
100 ms
inhibition [%]
50
25
0
1.0
3.0
bic [μM]
C
α2fl/fl
sns-α2-/-
DZP
control
wash
100 μV
100 ms
200 μV
100 ms
potentiation [%]
80
40
0
0.3
1.0
3.0
DZP [μM]
*
*
*

	Acute nociception		**Inflamed**		**antihyperalgesia by diazepam**	
	thermal (PWL, s)	***mechanical (PWT, g)***	***thermal (PWL, s)***	***mechanical (PWT, g)***	***thermal (AUC, s ɦ)***	***mechanical (AUC, g ɦ)***
wild-type	14.8 ± 0.8 (n = 7)	3.5 ± 0.06 (n = 9)	5.65 ± 0.19 (n = 7)	0.94 ± 0.10 (n = 9)	19.9 ± 2.9 (n = 7)	6.55 ±1.0 (n = 9)
sns-cre⁺	14.4 ± 2.6 (n = 8)	3.6 ± 0.09 (n = 7)	5.73 ± 0.91 (n = 8)	1.02 ± 0.06 (n = 7)	20.4 ±3.0 (n = 8)	6.34 ± 0.3 (n = 7)
P (unpaired t-test)	0.71	0.63	0.95	0.58	0.91	0.83

Tab. 4. Baseline nociceptive sensitivity, inflammatory hyperalgesia (48 h after subcutaneous zymosan A injection) and antihyperalgesic effect of diazepam (0.09 mg / kg, i.t.) in wild-type and sns-cre transgenic mice. Paw withdrawal latencies (PWL; s) in response to stimulation with defined radiant heat, mechanical thresholds (PWT; g) to stimulation with dynamic von Frey filaments in wild-type and *sns*-cre⁺ littermates. Antihyperalgesia was quantified as the area under the curve (AUC) of the change from before-drug baseline plotted versus time. All values mean ± sem.

	acute nociception			**inflammatory hyperalgesia / paw swelling**			**neuropathic hyperalgesia**		**capsaicin-induced sensitization**
	thermal (PWL, s)	***mechanical (PWT, g)***	***chemical (flinches / 5 min)***	***thermal (AUC, s ·d)***	***mechanical (AUC, g ·d)***	***paw swelling (AUC, ml ·h)***	***thermal (AUC, s ·d)***	***mechanical (AUC, g ·d)***	***mechanical (AUC, g ·h)***
$\alpha2^{fl/fl}$	15.0 ± 0.6 (n = 6)	3.0 ± 0.1 (n = 6)	49.5 ± 6.6 (n = 6)	33.3 ± 4.6 (n = 6)	9.1 ± 0.3 (n = 6)	3.62 ± 0.42 (n = 6)	224 ± 9 (n = 6)	47.3 ± 1.5 (n = 6)	4.6 ± 0.24 (n = 5)
***sns*-$\alpha2^{-/-}$**	14.5 ± 0.8 (n = 10)	3.1 ± 0.1 (n = 10)	49.0 ± 7.1 (n = 6)	33.0 ± 4.6 (n = 10)	10.6 ± 0.7 (n = 10)	3.25 ± 0.31 (n = 10)	230 ± 8 (n = 6)	44.8 ± 1.4 (n = 6)	4.5 ± 0.22 (n = 6)

Tab. 5. Baseline nociceptive sensitivity and inflammatory and neuropathic hyperalgesia in wild-type and *sns*-$\alpha2^{-/-}$ mice. Paw withdrawal latencies (PWL; s) in response to stimulation with defined radiant heat, mechanical thresholds (PWT; g) to stimulation with electronic von Frey filaments, and numbers of flinches within 5 min after subcutaneous injection of capsaicin (1.6 µg in 10 µl) in *sns*-$\alpha2^{-/-}$ mice and in wild-type ($\alpha2^{fl/fl}$) littermates. Hyperalgesia was quantified as the area under the curve (AUC) of the change from baseline sensitivity plotted versus time. $P>0.1$ (unpaired t test) for all comparisons between genotypes. All values mean ± sem.

When tested in an inflammatory pain model (subcutaneous injection of the yeast extract zymosan A into one hind paw), *sns*-α2$^{-/-}$ and α2$^{fl/fl}$ mice developed virtually extract zymosan A into one hind paw), *sns*-α2$^{-/-}$ and α2$^{fl/fl}$ mice developed virtually identical thermal and mechanical hyperalgesia and similar paw swelling (fig. 5A-C). Likewise, *sns*-α2$^{-/-}$ and α2$^{fl/fl}$ mice responded with nearly identical thermal and mechanical hyperalgesia after chronic constriction injury (CCI) of the left sciatic nerve (fig. 5D,E), and developed unchanged mechanical hyperalgesia after subcutaneous capsaicin injection (fig. 5F).

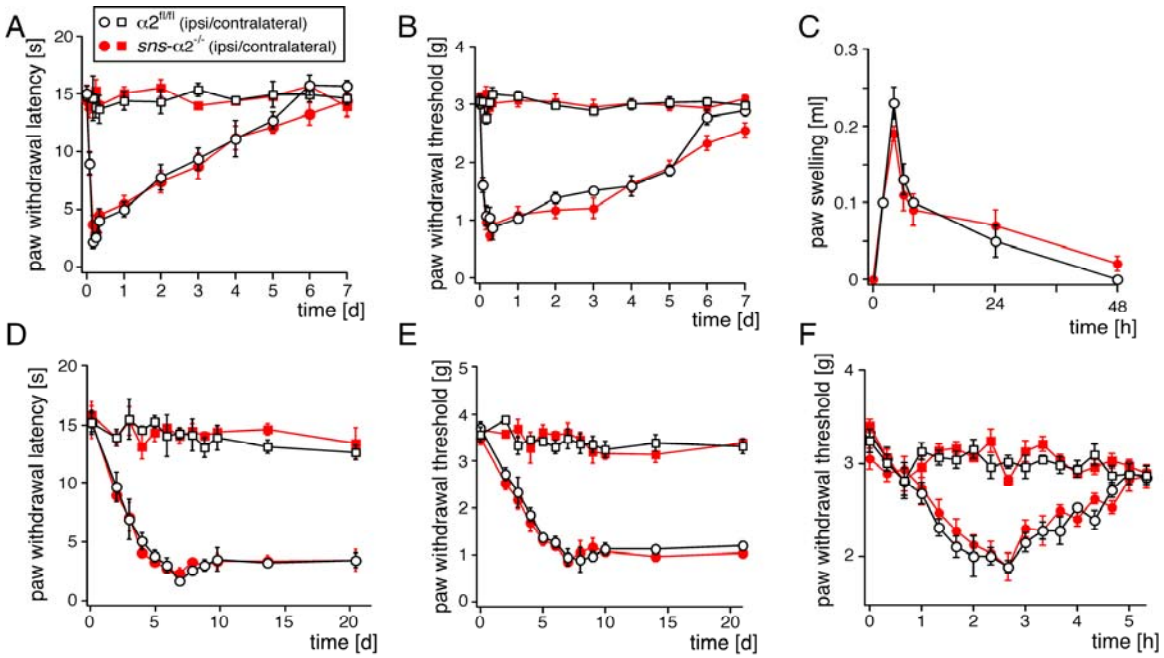


Fig. 5. Nociceptive behavior in *sns*-α2$^{-/-}$ mice. Inflammation induced by subcutaneous zymosan A injection (0.06 mg / 10 µl) into the plantar side of the left hind paw. Thermal hyperalgesia (paw withdrawal latencies, s) (A), mechanical sensitization (paw withdrawal thresholds, g) (B), and paw swelling (C) in *sns*-α2$^{-/-}$ and wild-type (α2$^{fl/fl}$) littermates. *n* = 6 - 10 mice / group. (D, E) Same as B, C, but neuropathic pain induced through CCI surgery of the left sciatic nerve. *n* = 6 mice / group. (F) Secondary hyperalgesia induced through subcutaneous injection of capsaicin (30 µg in 10 µl) into the plantar left hind paw. Mechanical withdrawal thresholds (g). *n* = 5 - 6 mice / group. For statistics see Tab. 5.

In separate experiments, we assessed the consequences of *sns*-α2 gene deletion for the antihyperalgesic effects of spinal DZP in inflammatory and neuropathic pain. DZP (0.09 mg/kg body weight, compare Knabl et al. (2008) was injected i.t. at the level of the lower lumbar spine. Injections were made 2 days after zymosan A injection and 7 days after CCI surgery, when inflammatory or neuropathic hyperalgesia had reached a maximum (for the time course of sensitization compare Reinold et al., 2005, and Hösl et al., 2006). DZP reversibly reduced thermal and mechanical hyperalgesia to similar degrees in both pain models. This antihyperalgesia was profoundly reduced in global α2 $GABA_A$ point mutated mice ($\alpha2^{R/R}$ mice) confirming the dominant contribution of α2 $GABA_AR$ (fig. 6). In the inflammatory pain model, the antihyperalgesic effect of i.t. DZP in *sns*-$\alpha2^{-/-}$ mice fell between those of wild-type ($\alpha2^{fl/fl}$) and $\alpha2^{R/R}$ mice for thermal and mechanical hyperalgesia indicating that presynaptic α2 $GABA_AR$ contributed significantly to α2 dependent antihyperalgesia (fig. 6A,B). Although i.t. DZP was similarly effective against neuropathic hyperalgesia and although this antihyperalgesia was also mainly mediated by α2 $GABA_AR$, neuropathic *sns*-$\alpha2^{-/-}$ mice responded normally to i.t. DZP (fig. 6C,D).

Because compensatory processes are of major concern in gene deletion studies (Rudolph and Möhler, 2004), we included nociceptor-specific α2 point mutated mice (*sns*-$\alpha2^{/R}$) in addition to *sns*-$\alpha2^{-/-}$ and $\alpha2^{R/R}$ mice in a subset of experiments (those on mechanical hyperalgesia; fig. 6B, D).

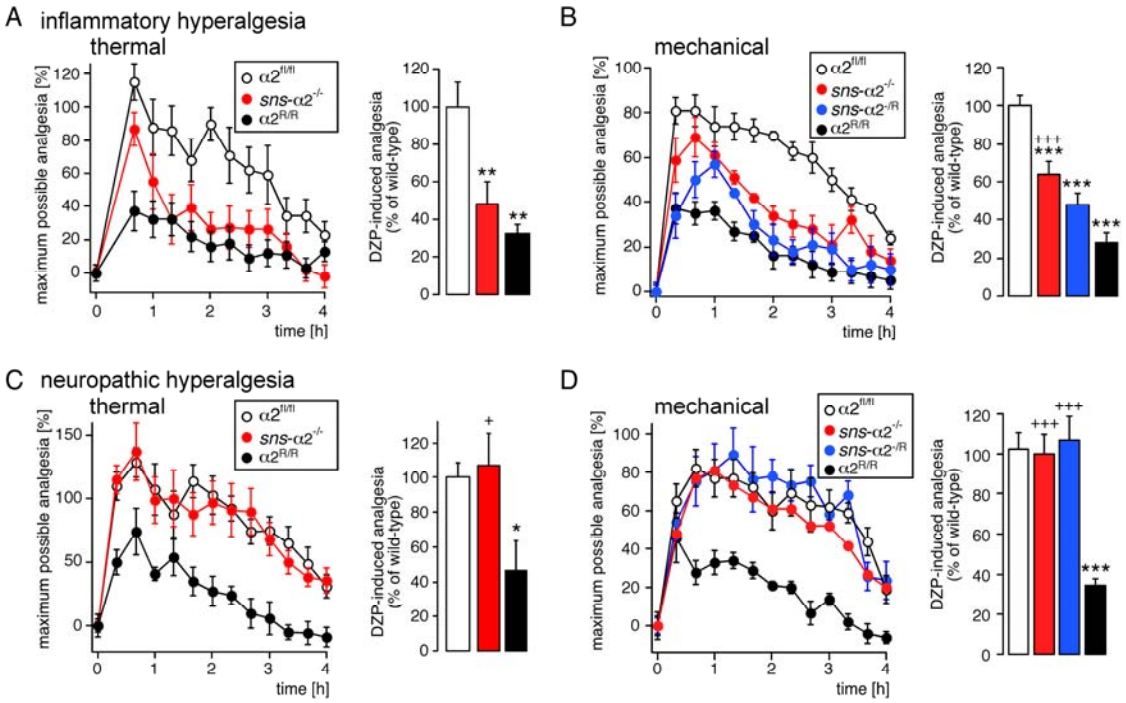


Fig. 6. Antihyperalgesic effects of DZP. Antihyperalgesic effects of intrathecally injected DZP (0.09 mg / kg body weight) on thermal (A, C) and mechanical (B, D) hyperalgesia expressed as percent maximum possible analgesia (mean ± sem). AUC (0 - 4 h after DZP injection). (A, B) Inflammatory hyperalgesia induced by subcutaneous zymosan A injection (0.06 mg in 10 µl) into the left hind paw. DZP was given 48 hours after zymosan A injection Left: time course; Right: statistics. AUC expressed as percent of wild-type littermates ($\alpha2^{fl/fl}$ mice). ANOVA F (2,25) = 8.71 followed by Bonferroni post hoc text, n = 8 - 10 mice / group (thermal hyperalgesia); ANOVA F (3,33) = 36.82, n = 7 - 12 mice / group (mechanical hyperalgesia). (C, D) Same as (A, B) but neuropathic pain 7 days after CCI surgery of the left sciatic nerve. ANOVA followed by Bonferroni post hoc test F (2,21) = 5.18, n = 7 - 9 mice / group (thermal hyperalgesia); F (3,23) = 11.16, n = 5 - 10 mice/group (mechanical hyperalgesia). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, significant against $\alpha2^{fl/fl}$, $^{+}$, $P \leq 0.05$; $^{+++}$, $P \leq 0.001$, against $\alpha2^{R/R}$.

These "tissue-specific point-mutated" mice carry a point-mutated and a floxed (wild-type) allele in all cells of the body with the exception of primary nociceptors which only express the mutated allele after cre-mediated deletion of the wild-type allele. In all tests performed, the phenotypes of these *sns*-α2 $^{/R}$ mice closely resembled those of *sns*-α2$^{-/-}$ mice. Because heterozygous nociceptor-specific α2 deficient (*sns*-α2 $^{-/+}$) mice and heterozygous α2 point mutated (α2$^{H/R}$) mice showed no behavioral changes compared to wild-type (α2$^{fl/fl}$) mice (Tab. 6), the phenotype of *sns*-α2 $^{/R}$ mice clearly originated from the presence of the point mutation in primary nociceptors. These experiments therefore render compensatory up-regulations of other DZP-sensitive $GABA_AR$ in the *sns*-α2$^{-/-}$ mice unlikely.

	Acute nociception (PWT, g)	**Inflammatory hyperalgesia (PWT, g)**	**Antihyperalgesia by diazepam (AUC, g·h)**
α2$^{fl/H}$ (n = 7)	3.59 ± 0.06	0.82 ± 0.11	7.80 ± 2.95
***sns*-α2$^{-/H}$(n = 6)**	3.55 ± 0.13	1.02 ± 0.08	7.59 ± 0.35
α2$^{R/H}$ (n = 6)	3.50 ± 0.11	0.99 ± 0.08	8.12 ± 0.63
P (ANOVA)	0.56	0.90	0.76

Tab. 6. Baseline mechanical sensitivity, inflammatory hyperalgesia and antihyperalgesic effect of diazepam (0.09 mg / kg, i.t.) in heterozygous nociceptor-specific a2-deficient (*sns*-α2$^{-/H}$) mice, heterozygous point mutated (a2$^{H/R}$) mice, and heterozygous a2-floxed (wild-type) mice. Antihyperalgesia was quantified as the area under the curve (AUC) of the change from before-drug baseline sensitivity plotted versus time. All three genotypes had virtually identical baseline mechanical sensitivities, developed similar mechanical hyperalgesia and responded normally to DZP (0.09 mg / kg i.t.). Because the floxed *Gabra2* allele behaves as a wild-type allele in the absence of cre expression, α2$^{fl/H}$ mice can be considered as wild-type mice. Hence, these experiments demonstrate that the histidine to arginine point mutation of only one *Gabra2* allele (α2$^{H/R}$ mice) has no apparent consequences for pain control or for the anti-hyperalgesic effect of intrathecal DZP. Similarly, the nociceptor specific deletion of one *gabra2* allele (in *sns*-α2$^{-/H}$ mice) affected neither baseline mechanical sensitivity nor the development of mechanical hyperalgesia or its reversal by intrathecal DZP. The phenotype described for the nociceptor specific α2 point mutated (*sns*-α2$^{-/R}$) mice can thus be specifically attributed to the lack of DZP sensitive α2 $GABA_AR$ in primary nociceptors. All values mean ± sem.

Discussion

Although presynaptic $GABA_AR$ have been extensively studied in various CNS areas (Kullmann et al., 2005), their roles in integrative CNS functions and as targets for GABAergic drugs have remained difficult to assess. Here, we have used a genetic approach to selectively interfere with presynaptic $GABA_AR$ on spinal nociceptor terminals and to investigate their contribution to spinal pain control. We used confocal double labeling experiments to study the expression pattern of α2 $GABA_AR$ in the spinal dorsal horn, electrophysiological recordings in spinal cord slices and isolated spinal cords to assess their contribution to the modulation of primary afferent-evoked synaptic transmission, and finally behavioral experiments to study their role in pain control.

Previous *in situ* hybridization (Persohn et al., 1991; Ma et al., 1993), immunofluorescence (Bohlhalter et al., 1996; Knabl et al., 2008), and electrophysiological (Knabl et al., 2008) experiments have suggested that $GABA_AR$ on primary sensory neurons are mainly, if not exclusively, of the α2 subtype. Our confocal double labeling experiments confirm the presence of α2 $GABA_AR$ on peptidergic and nonpeptidergic nociceptors as well as on non-nociceptive fibers. The additional presence of α3 subunits found in all three fiber types is consistent with our electrophysiological results, which demonstrate that GABAergic membrane currents in nociceptive DRG neurons and DRPs were still potentiated by DZP in *sns*-$\alpha2^{-/-}$ mice, albeit to a lesser extent than in wild-type mice.

GABAergic axo-axonic synapses onto the presynaptic terminals of primary afferent nerve fibers have been extensively investigated in monkey (Alvarez et al., 1993) and cat (Alvarez et al., 1992), but data in mice is spares. In monkey and cat electron microscopy studies, GABAergic terminals were found presynaptic to Aδ fiber terminals but not to C fiber terminals. Our study however provides clear evidence for the presence of $GABA_AR$ on the intra-spinal segments of peptidergic and nonpeptidergic C fibers in mice, and also for their functionality as ablation of α2 $GABA_AR$ in the *sns*-$\alpha2^{-/-}$ mice almost completely abolished the potentiating effect of DZP on DRPs. Although the *sns*-cre is active not only in C fiber nociceptors but also in Aδ nociceptors (Gangadharan et al., 2009), these actions cannot be ascribed to α2 $GABA_AR$ on Aδ fibers alone, because recent evidence indicates that in particular heat hyperalgesia is largely if not exclusively mediated by peptidergic C fibers (Abrahamsen et al., 2008; Cavanaugh et al., 2009). Provided that the absence in

monkey and cat of GABAergic terminals presynaptic to C fiber endings translates to mice, our findings may prompt for structural arrangements of the GABAergic input different from classical axo-axonic synapses. In such an alternative scenario, GABAergic inhibition of C fiber nociceptors might not originate from $GABA_AR$ located at the presynaptic terminal itself but from axonal receptors located farther away from the terminals. Such an arrangement would impair action potential propagation rather than directly interfere with transmitter release, and would be similar to what has been described for muscle spindle afferents in the rat brain stem (Verdier et al., 2003). These axonal receptors might become activated through ambient GABA rather than through GABA released directly onto these receptors.

The most obvious behavioral phenotype observed in *sns*-$\alpha2^{-/-}$ mice was a reduction in the antihyperalgesic effect of spinal DZP against inflammatory hyperalgesia. At least for inflammatory hyperalgesia, this phenotype unambiguously indicates that the antihyperalgesic action of spinal BDZ is largely due to a direct action on the sensory pain pathway and not due to indirect effects such as a reduction in anxiety-induced hyperalgesia. It also indicates that the enhancement on primary afferent depolarization by spinally applied BDZs increases presynaptic inhibition in primary nociceptors and thereby reduces nociceptive input to the spinal dorsal horn. Diminished DZP-induced antihyperalgesia in *sns*-$\alpha2^{-/-}$ mice correlates well with the decreased ability of DZP to facilitate $GABA_AR$-mediated inhibition of synaptic transmission between primary nociceptors and second order neurons, and with the diminished DZP-sensitivity of GABAergic DRPs in these mice. About one third of the α2 $GABA_AR$-mediated antihyperalgesia was maintained in *sns*-$\alpha2^{-/-}$ mice. This part may originate from α2 $GABA_AR$ expressed by intrinsic dorsal horn neurons. Expression of α2 $GABA_AR$ on intrinsic dorsal horn neurons has not been generally accepted previously, because *in situ* hybridization studies had revealed significant amounts of α2 mRNA in the ventral but not in the dorsal horn (Ma et al., 1993). Our experiments demonstrate that much of the α2 immunofluorescence is retained in *sns*-$\alpha2^{-/-}$ mice consistent with our previous electrophysiological data showing reduced DZP-sensitivity in dorsal horn neurons of $\alpha2^{R/R}$ mice (Knabl et al., 2008). Alternatively, the remaining α2 $GABA_AR$-mediated antihyperalgesia could come from α2 $GABA_AR$ residing on primary sensory neurons which do not express the *sns*-cre.

In contrast to the antihyperalgesic activity of spinal DZP against inflammatory pain, its activity against neuropathic pain was not changed in *sns*-α2$^{-/-}$ or *sns*-α2 $^{-/R}$ mice. It is tempting to speculate that presynaptic inhibition by α2 $GABA_AR$ might be less important under neuropathic conditions. However, Abrahamsen et al (2008) demonstrated that different types of primary afferent sensory fibers mediate inflammatory and neuropathic hyperalgesia. In fact, neuropathic hyperalgesia developed normally in mice lacking *sns*-positive primary nociceptors, whereas inflammatory hyperalgesia was largely abolished (Abrahamsen et al., 2008). It is therefore possible that the antihyperalgesic action of intrathecal diazepam against neuropathic pain also occurred through presynaptic α2 $GABA_AR$ but residing on primary afferent sensory fibers which did not express *sns*-cre.

$GABA_AR$ on spinal nociceptor terminals have been suggested to inhibit the transmission of nociceptive signals through PAD and subsequent presynaptic inhibition (Willis, 1999). The *sns*-α2$^{-/-}$ mice studied here had normal baseline nociceptive sensitivities and developed normal inflammatory or neuropathic hyperalgesia. Very intense nociceptor stimulation and inflammation may however enhance PAD to levels sufficient to trigger action potentials and to elicit so called dorsal root reflexes (Cervero and Laird, 1996; Willis, 1999). Input from primary afferent nerve fibers could then, via interconnected GABAergic interneurons, elicit action potentials in other primary afferent fiber terminals, from which excitation could spread both anterogradely and retrogradely, to exaggerate pain and inflammation. Again, *sns*-α2$^{-/-}$ mice exhibited unaltered hyperalgesia after capsaicin injection and unchanged hyperalgesia or paw swelling after zymosan A injection. Nevertheless, our findings do not exclude a contribution of GABAergic PAD to presynaptic inhibition or dorsal root reflexes, because the $GABA_AR$ remaining in nociceptors of *sns*-α2$^{-/-}$ mice were apparently sufficient to sustain GABAergic membrane currents and DRPs of nearly normal amplitude. Reduced BDZ sensitivity of $GABA_AR$ currents in nociceptive DRG neurons and of dorsal root potentials but nearly unchanged amplitudes and unaffected bicuculline sensitivity may be explained by the up-regulation of BDZ-insensitive $GABA_AR$ subunits. A significant up-regulation was found for α4. In addition, other BDZ-insensitive but bicuculline-sensitive subunits (δ and θ) showed a trend towards increased expression in *sns*-α2$^{-/-}$ mice. One might speculate that a facilitation of $GABA_AR$ -mediated dorsal root reflexes by BDZs could also counteract antihyperalgesia by spinal BDZs. However, although

DRPs in *sns*-$\alpha 2^{-/-}$ mice were less sensitive to DZP, these mice did not show increased antihyperalgesia.

In summary, the generation of mice with a genetic ablation of a specific $GABA_AR$ subtype in primary nociceptors allowed us to attribute to presynaptic $GABA_AR$ residing on the axons or terminals of primary nociceptors a significant role in spinal pain control, namely a contribution to antihyperalgesia mediated by spinal DZP.

Acknowledgements

This work has been supported in part by grants from the Swiss National Science Foundation (31003A-116064 and 31003A-131093/1) to H.U.Z and from the Canadian Institutes of Health Research (CIHR; Grant DOP-73908) to F.C. who holds a CIHR Research Chair. J.S.W. is a Fonds de la Recherche en Santé de Quebec (FRSQ) postdoctoral fellow. We thank Isabelle Camenisch and Albena Davidova for genotyping of mice.

References

Abrahamsen B, Zhao J, Asante CO, Cendan CM, Marsh S, Martinez-Barbera JP, Nassar MA, Dickenson AH, Wood JN (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. Science 321:702-705.

Agarwal N, Offermanns S, Kuner R (2004) Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia. Genesis 38:122-129.

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002) PGE_2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 5:34-40.

Alvarez FJ, Kavookjian AM, Light AR (1992) Synaptic interactions between GABA-immunoreactive profiles and the terminals of functionally defined myelinated nociceptors in the monkey and cat spinal cord. J Neurosci 12:2901-2917.

Alvarez FJ, Kavookjian AM, Light AR (1993) Ultrastructural morphology, synaptic relationships, and CGRP immunoreactivity of physiologically identified C-fiber terminals in the monkey spinal cord. J Comp Neurol 329:472-490.

Alvarez-Leefmans FJ (2009) Chloride Transporters in Presynaptic Inhibition, Pain and Neurogenic Inflammation. In: Physiology and Pathology of Chloride Transporters and Channels in the Nervous System (Alvarez-Leefmans FJ, Delpire E, eds), pp 439-470. Amsterdam: Elsevier.

Arbuckle JB, Docherty RJ (1995) Expression of tetrodotoxin-resistant sodium channels in capsaicin-sensitive dorsal root ganglion neurons of adult rats. Neurosci Lett 185:70-73.

Blair NT, Bean BP (2003) Role of tetrodotoxin-resistant Na^+ current slow inactivation in adaptation of action potential firing in small-diameter dorsal root ganglion neurons. J Neurosci 23:10338-10350.

Bohlhalter S, Weinmann O, Mohler H, Fritschy JM (1996) Laminar compartmentalization of $GABA_A$-receptor subtypes in the spinal cord: an immunohistochemical study. J Neurosci 16:283-297.

Bormann J, Hamill OP, Sakmann B (1987) Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. J Physiol 385:243-286.

Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A 106:9075-9080.

Cervero F, Laird JM (1996) Mechanisms of touch-evoked pain (allodynia): a new model. Pain 68:13-23.

Gangadharan V, Agarwal N, Brugger S, Tegeder I, Bettler B, Kuner R, Kurejova M (2009) Conditional gene deletion reveals functional redundancy of $GABA_B$ receptors in peripheral nociceptors in vivo. Mol Pain 5:68.

Hosl K, Reinold H, Harvey RJ, Muller U, Narumiya S, Zeilhofer HU (2006) Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor α3 subunit, which mediate central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection. Pain 126:46-53.

Ishikawa T, Marsala M, Sakabe T, Yaksh TL (2000) Characterization of spinal amino acid release and touch-evoked allodynia produced by spinal glycine or $GABA_A$ receptor antagonist. Neuroscience 95:781-786.

Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy J-M, Rudolph U, Möhler H, Zeilhofer HU (2008) Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. Nature 451:330-334.

Kontinen VK, Dickenson AH (2000) Effects of midazolam in the spinal nerve ligation model of neuropathic pain in rats. Pain 85:425-431.

Kullmann DM, Ruiz A, Rusakov DM, Scott R, Semyanov A, Walker MC (2005) Presynaptic, extrasynaptic and axonal $GABA_A$ receptors in the CNS: where and why? Prog Biophys Mol Biol 87:33-46.

Labrakakis C, Tong CK, Weissman T, Torsney C, MacDermott AB (2003) Localization and function of ATP and $GABA_A$ receptors expressed by nociceptors and other postnatal sensory neurons in rat. J Physiol 549:131-142.

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 93:5860-5865.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Ma W, Saunders PA, Somogyi R, Poulter MO, Barker JL (1993) Ontogeny of $GABA_A$ receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. J Comp Neurol 338:337-359.

Martinez-Gomez J, Lopez-Garcia JA (2005) Electrophysiological and pharmacological characterisation of ascending anterolateral axons in the in vitro mouse spinal cord. J Neurosci Methods 146:84-90.

Mohler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. J Pharmacol Exp Ther 300:2-8.

Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 60:243-260.

Pearce RJ, Duchen MR (1994) Differential expression of membrane currents in dissociated mouse primary sensory neurons. Neuroscience 63:1041-1056.

Persohn E, Malherbe P, Richards JG (1991) In situ hybridization histochemistry reveals a diversity of $GABA_A$ receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. Neuroscience 42:497-507.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338:582-585.

Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Muller U, Zeilhofer HU (2005) Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. J Clin Invest 115:673-679.

Roberts LA, Beyer C, Komisaruk BR (1986) Nociceptive responses to altered GABAergic activity at the spinal cord. Life Sci 39:1667-1674.

Rudolph U, Mohler H (2004) Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol 44:475-498.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ (2003) The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur J Neurosci 17:13-27.

Turecek R, Trussell LO (2001) Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. Nature 411:587-590.

Verdier D, Lund JP, Kolta A (2003) GABAergic control of action potential propagation along axonal branches of mammalian sensory neurons. J Neurosci 23:2002-2007.

Wieland HA, Luddens H, Seeburg PH (1992) A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Willis WD, Jr. (1999) Dorsal root potentials and dorsal root reflexes: a double-edged sword. Exp Brain Res 124:395-421.

Zeilhofer HU (2008) Loss of glycinergic and GABAergic inhibition in chronic pain--contributions of inflammation and microglia. Int Immunopharmacol 8:182-187.

3.2 Antihyperalgesic Actions of α2 $GABA_A$ Receptors Occur Through a Spinal Site of Action

[1]Jolly Paul, [1]Gonzalo E. Yévenes, [1]Dietmar Benke, [1]Alessandra Di Lio, [1]Florence Crestani, [1]Robert Witschi, [2]James Cook, [3]Uwe Rudolph, [1]Jean-Marc Fritschy, [1,4]Hanns Ulrich Zeilhofer

[1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland; and Neuroscience Center Zurich

[2]Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, 3210 North Cramer Street, Milwaukee WI 53211, USA

[3]Laboratory of Genetic Neuropharmacology, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02478, USA

[4]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology ETH Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland

Manuscript in preparation

Author contributions: J.P., J.-M.F. and H.U.Z. designed research; J.P., G.E.Y., D.B., R.W., and F.C., performed research; A.D.L., R.W., J.C., and U.R. contributed reagents/analytic tools; J.P., G.E.Y., D.B., F.C., J.-M.F., and H.U.Z. analyzed data; H.U.Z. wrote the paper.

Abstract

Enhancing GABAergic inhibition at the spinal level alleviates inflammatory and neuropathic pain through specific subtypes of $GABA_A$ receptors ($GABA_AR$). Most of these $GABA_AR$ contain the $GABA_AR$ subunit α2. Their specific activation should evoke profound antihyperalgesia while avoiding most of the unwanted effects of classical benzodiazepine (BDZ) site agonists. While the relevance of spinal $GABA_AR$ in this process is firmly established, possible contributions of supraspinal receptors are less clear. These might exert a genuine antihyperalgesic action similar to that of spinal $GABA_AR$ or might contribute to pain relief through reversing anxiety-induced hyperalgesia. To address these potential roles, we generated two lines of $GABA_AR$ mutated mice which either lack α2 $GABA_AR$ specifically in the spinal cord and in all primary sensory neurons up to the mid cervical level, or which carry only BDZ-insensitive α2 $GABA_AR$ at these sites. We analyzed the consequences of these mutations for antihyperalgesia evoked by systemic treatment with the novel non-sedative BDZ site agonist HZ166 in the chronic constriction injury model of neuropathic pain. Wild-type mice and both types of mutated mice did not differ in their baseline nociceptive thresholds and developed similar neuropathic hyperalgesia. However, the antihyperalgesic responses to systemic HZ166 were reduced in both mutated mouse lines by about 70% and virtually indistinguishable from that of point-mutated mice, in which all α2 $GABA_AR$ had been rendered BDZ-insensitive. These results indicate that the antihyperalgesic actions of systemically administered BDZ site ligands do not require supraspinal α2 $GABA_AR$ and are thus independent of anxiolysis.

Introduction

Chronic neuropathic pain syndromes are frequently unresponsive to classical analgesic drugs including cyclooxygenase inhibitors and opioids. Drugs most effective in these pain conditions include anticonvulsants that modulate or block voltage-gated Na^+ or Ca^{2+} channels (Sang and Hayes, 2006). Other anticonvulsive drugs with a different mode of action include the benzodiazepine (BDZ) site agonists, which enhance neuronal inhibition through a facilitation of $GABA_AR$ -mediated neurotransmission. Diminished GABAergic inhibition at the spinal cord level has in fact been shown to be a major contributor to chronic pain syndromes (Ahmadi et al., 2002; Coull et al., 2003) suggesting that drugs facilitating spinal inhibition might indeed correct a major component of the maladaptive neuroplasticity underlying chronic pain (Zeilhofer, 2008; Munro et al., 2009). In line with this concept, previous work has shown that spinal injection of BDZ site agonists provide pain relief in a number of rodent models of inflammatory and neuropathic pain (Luger et al., 1995; Kontinen and Dickenson, 2000; Knabl et al., 2008). However, although anecdotal reports suggest some efficacy of classical BDZ in chronic pain patients, classical BDZ are usually not considered as first line therapeutics in these patients (Jasmin et al., 2004).

Mammalian $GABA_AR$ form a heterogeneous family of heteropentameric ion channels composed from a repertoire of 19 subunits. The different $GABA_AR$ subtypes are best characterized by the type of α subunit present in the individual receptors (Olsen and Sieghart, 2008). Experiments in genetically modified mice demonstrated a particular relevance of $GABA_AR$ containing α2 and α3 subunits for antihyperalgesia mediated by spinally applied BDZ (Knabl et al., 2008). These and subsequent experiments (Knabl et al., 2009) from our group established that the antihyperalgesic actions of BDZ site agonists occur independent of the sedative actions mediated by α1 containing $GABA_AR$ (Rudolph et al., 1999). More recent experiments with novel BDZ site ligands with improved subunit specificity (i.e. reduced or even absent activity α1 $GABA_AR$) have shown that such novel compounds are able to reduce nerve injury and inflammation-induced hyperalgesia after systemic administration in the absence of sedation (Knabl et al., 2008; Munro et al., 2008; Knabl et al., 2009; Munro et al., 2009; Di Lio et al., 2011). While the contribution of spinal $GABA_AR$ to this antihyperalgesia has been studied in detail, much less is known about a possible role of supraspinal $GABA_AR$. These receptors

might contribute to antihyperalgesia through a genuine antihyperalgesic effect, e.g. through $GABA_AR$ in the rostral agranular insular cortex (Jasmin et al., 2003), or through the reversal of anxiety-induced hyperalgesia (Andre et al., 2005). The latter possibility is particularly interesting as α2 and α3 $GABA_AR$ similarly contribute to antihyperalgesia and anxiolysis (Low et al., 2000; Morris et al., 2006). On the other hand, supraspinal $GABA_AR$ might partially counteract spinal antihyperalgesia for example through inhibiting antinociceptive tracts descending from the periaqueductal grey (PAG) or the rostroventromedial medulla (RVM) (Harris and Westbrook, 1995; Luger et al., 1995; Tatsuo et al., 1999). The subtypes of the $GABA_AR$ relevant to pain control at all these supraspinal sites are not known. It is thus possible that activation of supraspinal $GABA_AR$ either facilitates or impedes spinal antihyperalgesia.

To address these questions, we generated two lines of $GABA_AR$ -mutated mice. The first of these lines (*Hoxb8*-$\alpha2^{-/-}$) carries a tissue-specific deletion of the α2 $GABA_AR$ subunit from all spinal neurons, astrocytes and primary sensory neurons up to the mid cervical level (about C4). This tissue-specific ablation was achieved by crossing mice which carried a $GABA_AR$ α2 (*Gabra2*) allele flanked by loxP sites ($\alpha2^{fl}$ (Witschi et al., 2011)) with mice expressing the cre recombinase under the transcriptional control of the *Hoxb8* homeobox gene (Witschi et al., 2010). The second line can be viewed as a tissue-specific point-mutated α2 $GABA_AR$ mouse line (*Hoxb8*-$\alpha2^{R/-}$). This line carried the $\alpha2^{fl}$ allele, a BDZ-insensitive H101R point-mutated allele ($\alpha2^{R}$; (Low et al., 2000)), and the *Hoxb8*-cre transgene. At supraspinal sites, this line expresses the point-mutated allele together with the fully functional ("wild-type") $\alpha2^{fl}$ allele, while in the peripheral and spinal nervous system only the point-mutated allele is expressed. For pharmacological analyses, we used the novel non-sedative partial BDZ site agonist HZ166 (Rivas et al., 2009; Di Lio et al., 2011). Analysis of the antihyperalgesic effects of HZ166 in the two mutated mouse lines and comparison of these effects with those obtained in wild-type mice and in mice, in which all α2 $GABA_A$ had been rendered BDZ-insensitive, revealed that activation of supraspinal α2 $GABA_AR$ did not contribute to the antihyperalgesic actions of HZ166.

Materials and Methods

Generation of $GABA_A$ receptor mutated mice

Hoxb8-α2 $^{-/-}$ and *Hoxb8*-$\alpha2^{R/-}$ mice were generated by crossing $\alpha2^{fl/fl}$ mice (Witschi et al., 2011) and $\alpha2^{R/fl}$ mice with mice which carried in addition the *Hoxb8*-cre transgene (Witschi et al.). All mice were maintained at a C57BL/6J background. During the breeding of *Hoxb8*-α2 $^{-/-}$ mice, a small number of mice were born carrying a non-conditional loss of the $GABA_AR$ α2 allele which had probably occurred through undesired recombination events in the germ line. To ensure that these mice were excluded from further breeding and experiments we verified in all mice the absence of a knock-out allele in genomic DNA obtained from ear biopsies (where the *Hoxb8*-cre is not expressed during any developmental stage).

Cell culture and transfection

HEK 293 cells were maintained in DMEM supplemented with 10% FBS and seeded to a density of 500,000 cells onto polylysine-coated 100 mm culture dishes one day before transfection. Cells were transfected with plasmids containing the subunit combination α2β3γ2 or α2(H101R) β3γ2 (7 mg total DNA, ratio 1:1:2) using jetPEI transfection reagent (Polyplus-transfection). Twenty-four hours after transfection, HEK 293 cells were harvested in PBS and used for [^{3}H]Ro 15-4513 binding.

[^{3}H]Ro 15-4513 binding assay

For [^{3}H]Ro 15-4513 binding, HEK 293 cells were homogenized in 10 vol. 10 mM Tris pH 7.5, 0.32 sucrose, protease inhibitor cocktail (complete Mini, Roche Applied Science) and centrifuged at 1,000 g for 10 min at 4°C. The supernatant was carefully removed and centrifuged again for 20 min at 25,000 g at 4°C. The crude membrane pellet was resuspended in 10 mM Tris-HCl pH 7.4, protease inhibitor cocktail and washed once by centrifugation and resuspension. Aliquots of the crude membranes prepared from HEK293 cells expressing the α2β3γ2 or α2(H101R)β3γ2 subunit combination (150-200 mg protein) were incubated with increasing concentrations of HZ-166 (10^{-8}-10^{-4}M) and 6.3 nM [^{3}H]Ro 15-4513 (22.7 Ci/mmol, PerkinElmer) in a total volume of 200 µl for 90 min. on ice. Subsequently, the samples were filtered onto glass fiber filters using a 12-channnel semiautomated cell harvester (Scatron) and washed with ice-cold buffer (10

mM Tris-HCl pH 7.4). Non-specific [^{3}H]Ro 15-4513 binding was measured using 10 mM flumazenil. The radioactivity of the filters was determined by liquid scintillation counting using a Tricarb 2500 liquid scintillation analyzer. Binding data were analyzed using the GraphPad Prism software (version 5.02, GraphPad Software, USA).

Cell Culture, transfection and cDNA constructs

HEK 293 cells (CRL-1573; American Type Culture Collection, Manassas, VA, USA) were cultured using standard methods. The cells were transfected with α2/ α2(H101R), β3 and γ2 rat $GABA_AR$ expression vectors (28) using lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). The transfection mixture was composed of (in μg): 1 α2/β3, 3 γ2 and 0.5 EGFP. Expression of EGFP was used as a marker of successfully transfected cells. All recordings were made 18 – 36 hours after transfection.

Electrophysiology

The effects of HZ166 on $GABA_AR$ were studied in electrophysiological experiments on HEK293 cells transiently expressing $GABA_AR$. GABA-evoked currents were recorded in the whole-cell patch-clamp configuration at room temperature (20–24°C) at a holding potential of -60 mV. Patch electrodes were pulled from borosilicate glass and were filled with (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.4), 4 MgCl2, 0.5 GTP and 2 ATP. The external solution contained (in mM) 150 NaCl, 10 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. Recordings were performed with a HEKA EPC-7 amplifier and Patch Master v2.11 software (HEKA Elektronik, Lambrecht-Pfalz, Germany). GABA was applied to the recorded cell using a manually controlled pulse (4-6 s) of a sub-saturating GABA concentration (EC_5) using an outlet tube (200 μm ID) of a custom-designed gravity-fed micro perfusion system positioned 50-120 μm away from the recorded cell. The corresponding EC_5 values for wild-type α2β3γ2 or mutant α2(H101R)β3γ2 receptors were obtained experimentally after successive application of 0.1, 1, 5, 10, 30, 100, 300 and 500 μM GABA. The concentration-response curve parameters (EC_{50} and Hill coefficients, n_h) were obtained from the curve fits of normalized concentration–responses to the equation $I_{GABA} = I_{max}\ [GABA]n_h\ /\ ([GABA]n_h + [EC_{50}]n_h)$. The mean maximal current (I_{max}) indicated the average maximal current elicited by a concentration of 500 mM GABA. HZ166 was first dissolved in DMSO and subsequently diluted into the recording solution on the day of the experiment. HZ166 was co-applied together with GABA without preincubation.

Behavioral testing

Experiments were performed in 7 - 10 week old mice of both sexes. Care was taken to ensure that equal numbers of male and female mice were used in all experiments since no gender differences were expected. Unilateral constriction injury of the left sciatic nerve just proximal to the trifurcation was performed as described previously (Bennett and Xie, 1988; Hosl et al., 2006). Anesthesia was induced and maintained by 2% isoflurane, combined with oxygen (30%). The sciatic nerve was exposed at the mid-thigh level proximal to the sciatic trifurcation by blunt dissection through the biceps femoris muscle. About 5 mm of the nerve were freed of adhering connective tissue and three chromic gut ligatures (4/0) (Ethicon) were loosely put around the nerve with about 1 mm spacing. The ligatures were tied until they elicited a brief twitch in the hind limb. The surgical wound was closed in layers. Mechanical sensitivities were assessed before surgery and after 7 days when sensitization had reached its maximum. On day 7, the antihyperalgesic effects of HZ166 were assessed. Mechanical sensitivity was determined as the threshold mechanical force (g) that evoked an immediate withdrawal response upon stimulation of the plantar side of one hind paw with electronic von Frey filaments (IITC, Woodland Hills, CA). 4 - 5 measurements were made for each time point and animal. Measurements of paw withdrawal thresholds of the injured paw and of the contralateral paw were made alternately. Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 135/2009).

Results

The cre/loxP system was employed to generate mice lacking the $GABA_AR$ α2 subunit from the peripheral nervous system and from the spinal cord (*Hoxb8*-α2 $^{/-}$ mice), and point-mutated (*Hoxb8*-$\alpha2^{R/-}$) mice, whose peripheral and spinal $GABA_AR$ were rendered BDZ-insensitive (for details of the breeding scheme and for abbreviations of the genotypes see Tab. 1). *Hoxb8*-α2 $^{/-}$ mice carried a *Hoxb8*-cre transgene and two floxed $GABA_AR$ α2 ($\alpha2^{fl}$) alleles, while *Hoxb8*-$\alpha2^{R/-}$ mice carried the *Hoxb8*-cre transgene together with a $\alpha2^{fl}$ allele and a point-mutated (H101R) $GABA_AR$ α2 ($\alpha2^{R}$) allele.

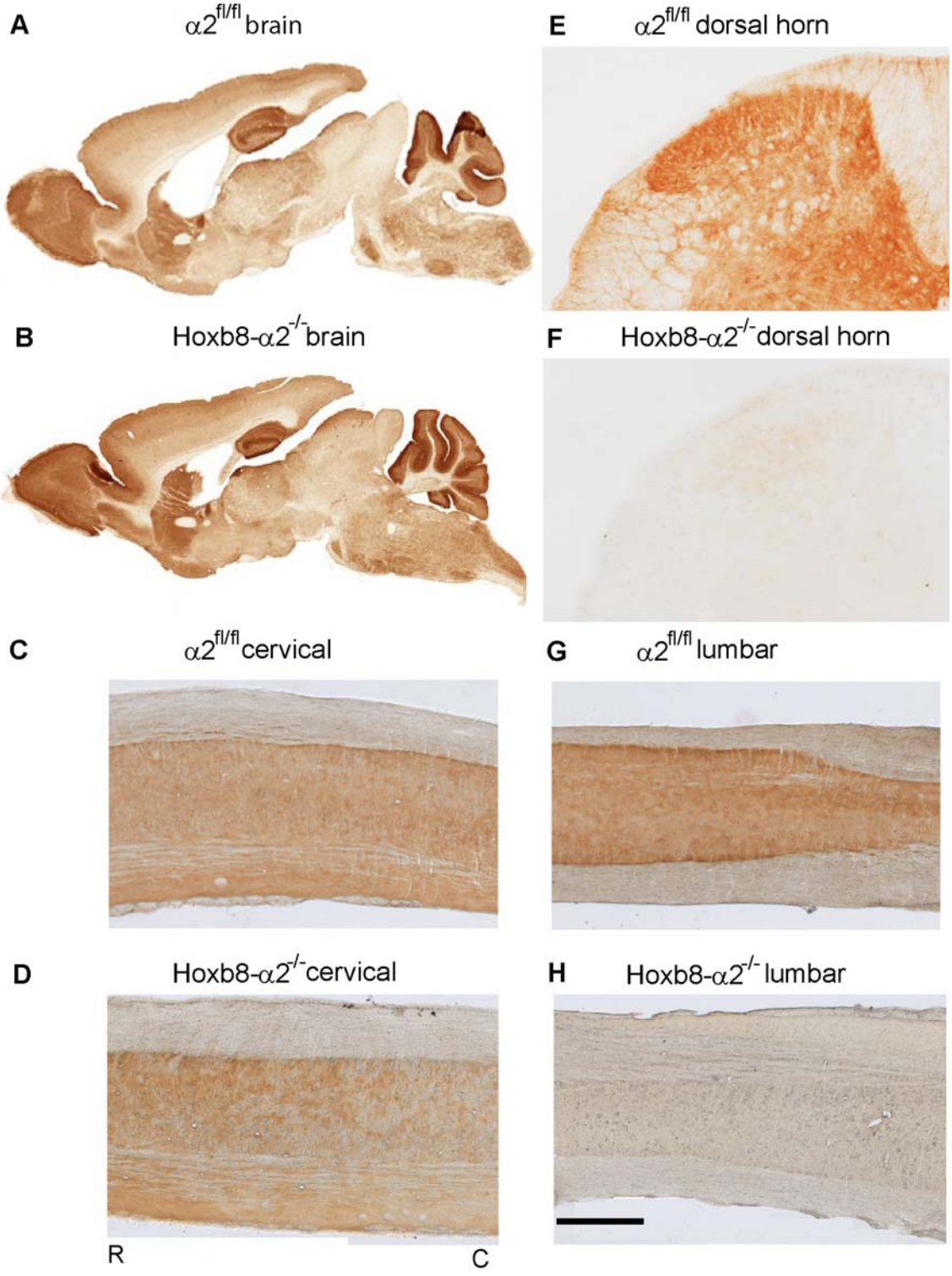
Morphological analyses demonstrated that the supraspinal $GABA_AR$ α2 subunit distribution of *Hoxb8*-α2 $^{/-}$ mice was indistinguishable from that of *Hoxb8*-$\alpha2^{fl/fl}$ (wild-type) mice (fig. 1). Sagittal sections of the cervical spinal cord revealed the expected progressive rostral to caudal loss of $GABA_AR$ α2 subunits within the upper cervical segments. Transverse sections of the lower lumbar spinal cord did not show any $GABA_AR$ α2 immunoreactivity, indicating highly effective gene *Hoxb8* cre-mediated recombination. The apparent lack of α2 $GABA_AR$ from the lumbar spinal cord of *Hoxb8*-α2 $^{/-}$ mice indicates that only few, if any, spinal α2 $GABA_AR$ resided on processes of neurons descending from supraspinal areas. Conversely, unchanged $GABA_AR$ α2 subunit expression in the brain indicates that neurons projecting from the spinal cord to supraspinal CNS areas made only a minor contribution to α2 $GABA_AR$ in the brain.

To investigate the consequences of these two mutations for the antihyperalgesic action of BDZ site ligands, we used the novel non-sedative partial BDZ site agonist HZ166, which has an improved side effect profile compared to classical BDZ site ligands. Before we analyzed the antihyperalgesic actions of this compound in *Hoxb8*-α2 $^{/-}$ and *Hoxb8*-$\alpha2^{R/-}$ mice, we verified that H101R α2 $GABA_AR$ currents were insensitive to modulation by HZ166. It has previously been shown that this point mutation dramatically reduces the binding of diazepam to $GABA_AR$ and completely abolishes their facilitation by diazepam (Wieland et al., 1992). This mutation can however have different effects on the action of other BDZ site ligands. The potentiating effect of bretazenil for example is enhanced in the point mutated receptors and the action of Ro 45-1513 is converted from negative to positive modulation (Benson et al., 1998).

genotype			***Gabra2* phenotype**		
Gabra2 allele 1	*Gabra2* allele 2	*Hoxb8*-cre transgene	Spinal sites	Supraspinal sites	designation (short)
fl	fl	-	wt / wt	wt / wt	$\alpha2^{fl/fl}$_*Hoxb8*-cre^{tg-} ($\alpha2^{fl/fl}$)
fl	fl	+	- / -	wt / wt	$\alpha2^{fl/fl}$_ *Hoxb8*-cre^{tg+} (*Hoxb8*-$\alpha2^{-/-}$)
fl	R	-	wt / R	wt / R	$\alpha2^{fl/R}$_ *Hoxb8*-cre^{tg-} ($\alpha2^{fl/R}$)
fl	R	+	R/-	wt / R	$\alpha2^{fl/R}$_ *Hoxb8*-cre^{tg+} (*Hoxb8*-$\alpha2^{R/-}$)
R	R	-	R / R	R / R	($\alpha2^{R/R}$)

Tab. 1: Genotypes and cell type-specific phenotypes of the mouse lines analyzed. Phenotypically, floxed alleles are regarded as wild-type in the absence of *cre*.

Fig. 1. Distribution of α2 $GABA_AR$ in the brain and spinal cord of wild-type ($\alpha2^{fl/fl}$) and *Hoxb8*-$\alpha2^{-/-}$ mice. Immunoperoxidase immunoreactivity (IR) for the $GABA_AR$ α2 subunit in brain and spinal cord. The sagittal brain sections from $\alpha2^{fl/fl}$ (**A**) and *Hoxb8*-$\alpha2^{-/-}$ (**B**) were stained for α2 subunit IR and no difference in distribution was apparent (n = 3 each genotype). In the cervical sections the rostral regions of both genotype had similar intensity (**C, D**) whereas caudally *Hoxb8*-$\alpha2^{-/-}$ mice showed a decrease in IR. The staining for α2 subunit was normal in the lumbar sections of $\alpha2^{fl/fl}$ mice but was absent in the lumbar sections of *Hoxb8*-$\alpha2^{-/-}$ mice (**E-H**). E-F: the coronal plane, G-H: the sagittal plane. Scale bar, 100μm.


A
α2fl/fl brain
E
α2fl/fl dorsal horn
B
Hoxb8-α2-/- brain
F
Hoxb8-α2-/- dorsal horn
C
α2fl/fl cervical
G
α2fl/fl lumbar
D
Hoxb8-α2-/- cervical
H
Hoxb8-α2-/- lumbar
R
C

We therefore compared the binding affinity of HZ166 to wild-type α2/β3/γ2 and point-mutated α2(H101R)/β3/γ2 $GABA_AR$. We found that the introduction of the H101R point mutation almost completely abolished binding of HZ166 to α2/β3/γ2 $GABA_AR$ (fig. 2). Only at a very high HZ166 concentration (100μM) Ro 45-1513 binding was marginally affected (26±6%).

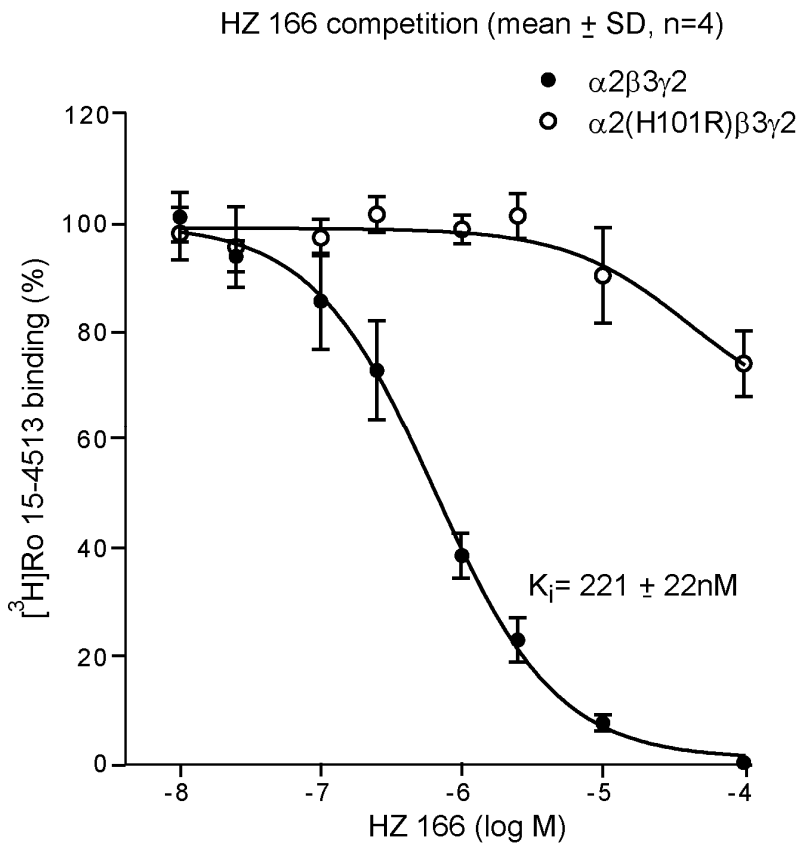
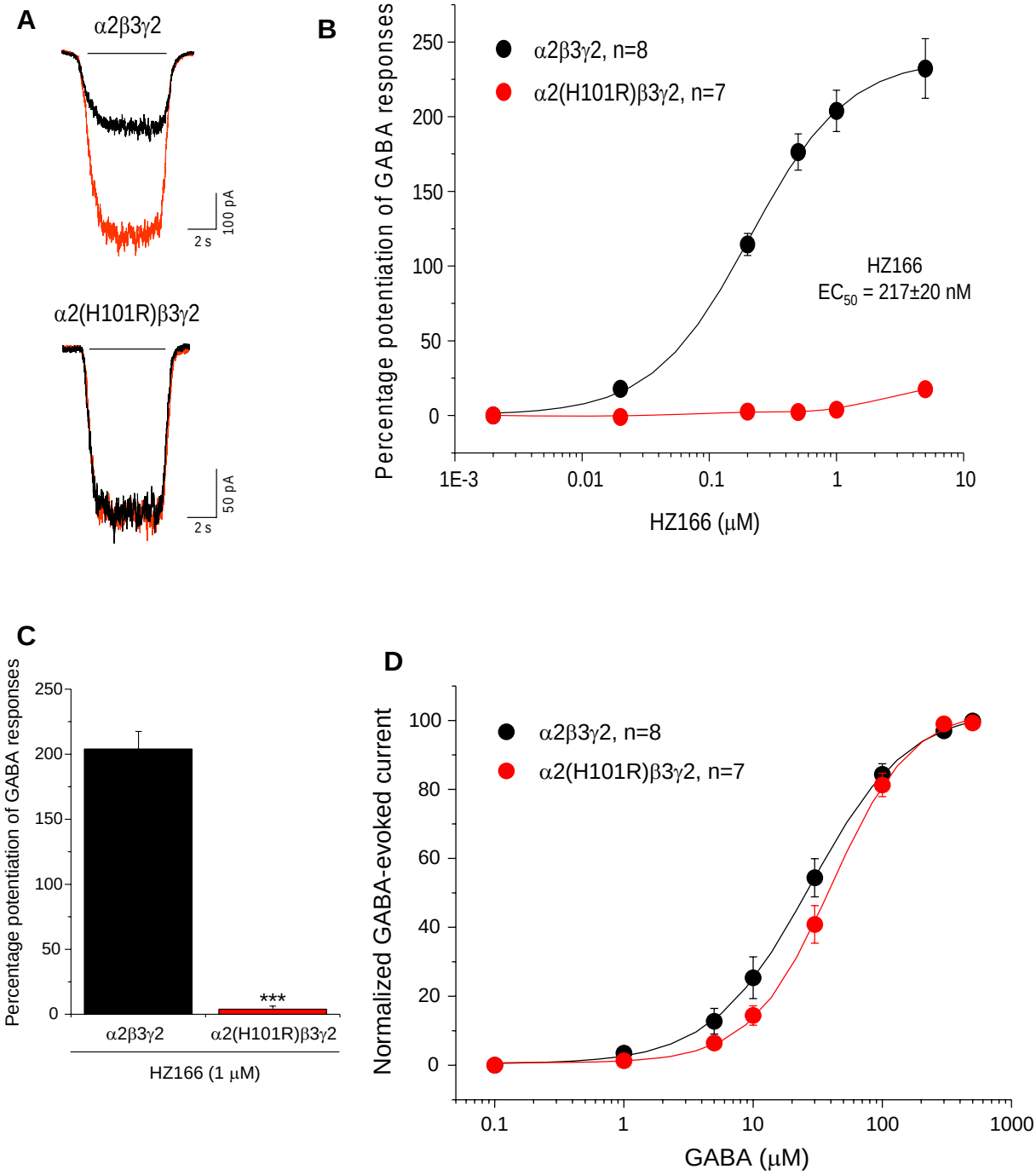


Fig. 2. Binding of HZ166 to wild-type and H101R point mutated α2 $GABA_AR$. Inhibition of [^{3}H]Ro15-4513 binding to HEK293 cell transfected with either the α2β2γ2 (●) or α2(H101R)β3γ2 subunit combination (○) by HZ166. Data represent the means ± standard deviation of 4 independent experiments. Error bars smaller than the symbol are not depicted.

Fig. 3. Potentiation of GABA-evoked currents by HZ166 on wild-type and α2(H101R)-mutated $GABA_AR$. **(A)** Examples of current traces through wild-type α2β3γ2 or mutated α2(H101R)β3γ2 $GABA_AR$ in absence (black) or presence of HZ166 (1 μM, red) **(B)** Sensitivity to HZ166 of the normalized GABA-activated currents in wild-type and α2(H101R)-mutated $GABA_AR$ using equipotent (EC_5) GABA concentrations **(C)** Summary of the current potentiation elicited by HZ166 in wild-type and α2(H101R)-mutated $GABA_AR$. The potentiation was largely attenuated by the mutation (***, P<0.001). (D) Sensitivity to GABA of recombinant α2β3γ2 or α2(H101R)β3γ2 receptors expressed in HEK cells.

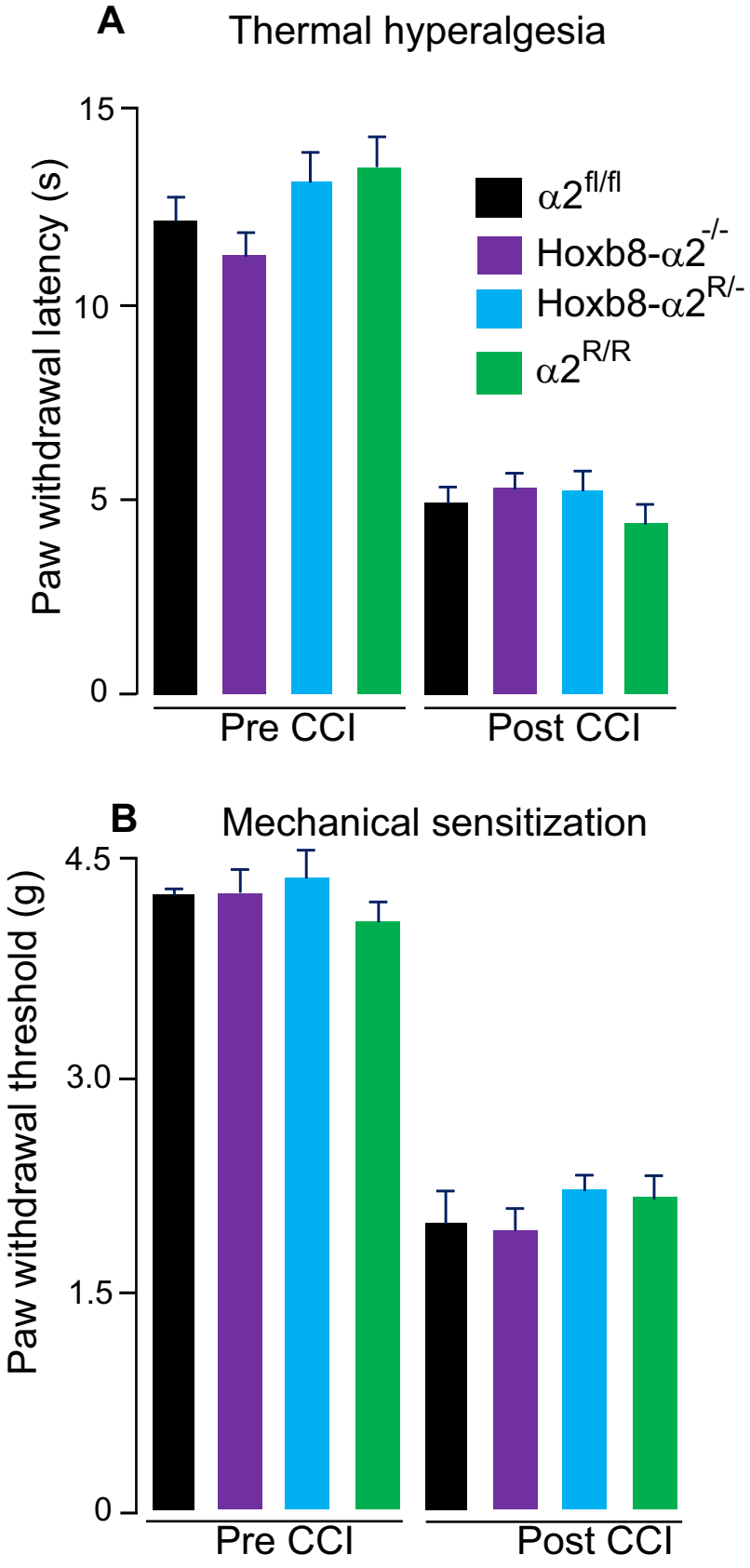


	EC_{50} (μM)	n_h	I_{max} (pA)	n
α2β3γ2	27±1	1.2±0.03	3659±479	8
α2(H101R)β3γ2	40±2	1.4±0.07	3489±693	7

Accordingly, electrophysiological experiments in HEK-293 cells transfected with either wild-type or mutated α2/β3/γ2 $GABA_AR$ showed that HZ166 had completely lost its ability for positive allosteric modulation in the point-mutated receptors (fig. 3). While HZ166 (1μM) potentiated GABA (EC5) evoked currents through wild-type α2/β3/γ2 $GABA_AR$ by 200%, potentiation was virtually absent in the point-mutated receptors.

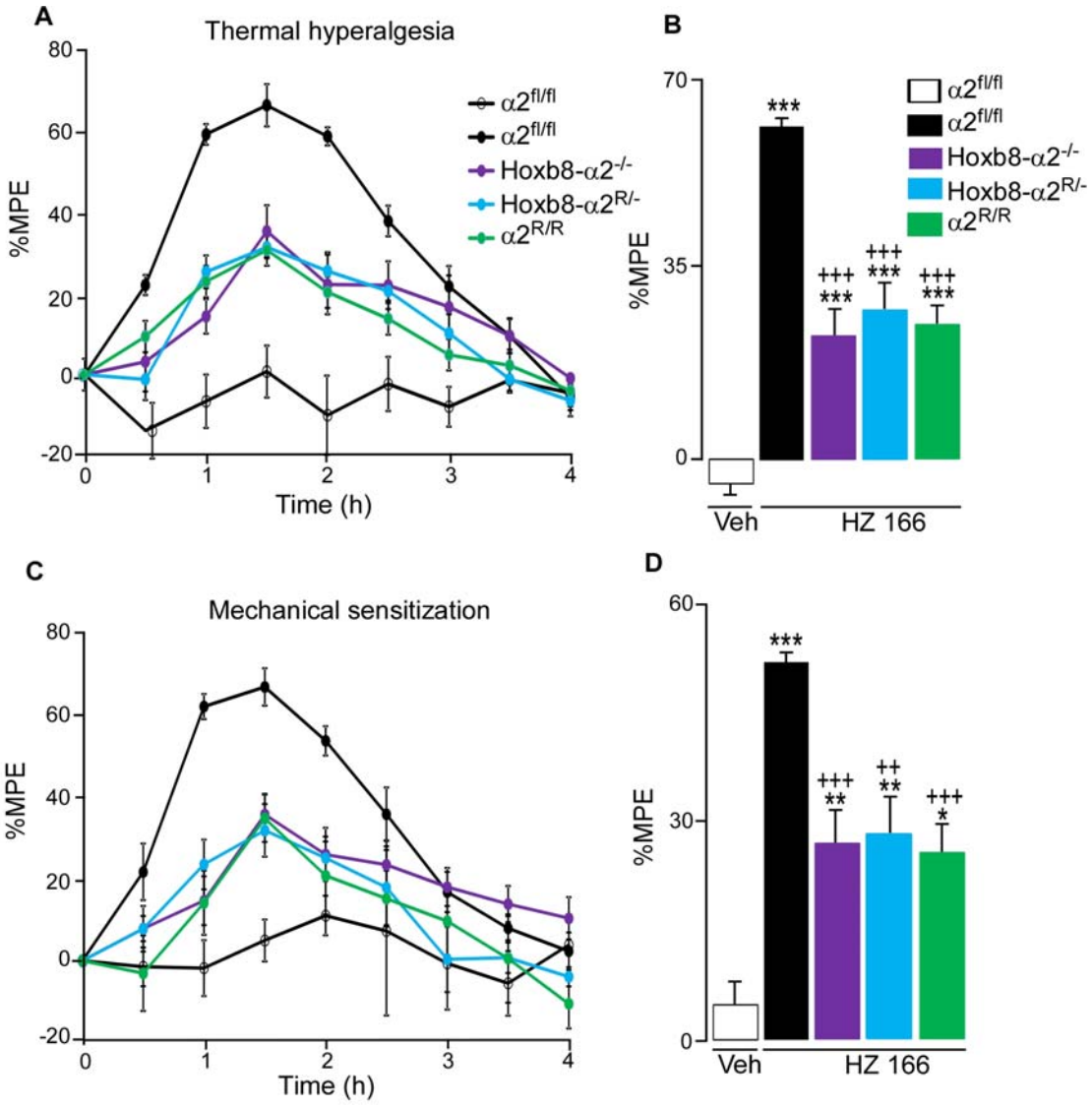
We then went on to investigate *Hoxb8*-$\alpha2^{-/-}$ and *Hoxb8*-$\alpha2^{R/-}$ mice in behavioral experiments and to address the susceptibility of these mice to antihyperalgesia by systemic HZ166. In this series of experiments we included in addition to *Hoxb8*-$\alpha2^{-/-}$ and *Hoxb8*-$\alpha2^{R/-}$ mice also wild-type ($\alpha2^{fl/fl}$) mice and homozygous α2 $GABA_AR$ H101R point-mutated mice ($\alpha2^{R/R}$). Comparison of wild-type with $\alpha2^{R/R}$ mice allowed us to determine the total contribution of α2 $GABA_AR$ to antihyperalgesia by HZ166. *Hoxb8*-$\alpha2^{-/-}$ mice, *Hoxb8*-$\alpha2^{R/-}$ mice and $\alpha2^{fl/fl}$ (wild-type) mice responded similarly to mechanical stimulation of their hind paws with electronic von Frey filaments and to thermal stimulation with a defined radiant heat source in the plantar test. Neuropathic pain sensitization induced by chronic constriction injury of the sciatic nerve developed similarly in (insert 'in') all genotypes (fig. 4).

Fig. 4. Baseline nociceptive sensitivity. Neuropathic pain induced by chronic constriction injury (CCI) to the sciatic nerve of the left hind paw. (**A**) Thermal hyperalgesia (paw withdrawal latencies, s), and (**B**) mechanical sensitization (paw withdrawal thresholds, g) in wild-type ($\alpha2^{fl/fl}$), *Hoxb8*-$\alpha2^{-/-}$, and *Hoxb8*-$\alpha2^{-/-}$ mice. Pre CCI and post CCI values are compared. n = 6 –10 mice / group. ANOVA $F(df)$= 1.4845 (pre CCI) and F(df) = 0.7107 (post CCI) for thermal hyperalgesia. ANOVA $F(df)$ = 0.7242 (pre CCI) and $F(df)$ = 0.5666 (post CCI) for mechanical hyperalgesia.


A
Thermal hyperalgesia
Paw withdrawal latency (s)
15
10
5
0
α2fl/fl
Hoxb8-α2-/-
Hoxb8-α2R/-
α2R/R
Pre CCI
Post CCI
B
Mechanical sensitization
Paw withdrawal threshold (g)
4.5
3.0
1.5
0
Pre CCI
Post CCI

No significant differences were observed in the degree of sensitization reached 7 days after surgery between the different genotypes for either mechanical or heat stimuli. We next compared the antihyperalgesic potential of HZ166 in the three types of mutant mice (*Hoxb8*-α2 $^{-/-}$, *Hoxb8*-α2$^{R/-}$ and α2$^{R/R}$ mice) with that in wild-type α2$^{fl/fl}$ mice. A dose of 16 mg/kg body weight applied intraperitoneally (i.p.) was chosen because this dose has previously been shown to produce a maximal antihyperalgesic response in the absence of sedative actions (Di Lio et al., 2011). A maximal antihyperalgesic response was observed between 60 and 90 minutes after drug injection. In wild-type mice, HZ166 reduced mechanical and thermal hyperalgesia by 61% and 52%, respectively (fig. 5). Both the time course and the degree of antihyperalgesia were very close to those reported previously by our group for wild-type C57BL/6 mice (Di Lio et al., 2011). This maximal possible effect was reduced in α2$^{R/R}$ mice to 25% indicating that about 60% of the antihyperalgesic actions of HZ166 were mediated by α2 $GABA_AR$. The degree of antihyperalgesia observed in both *Hoxb8*-α2$^{-/-}$ and *Hoxb8*-α2$^{R/-}$ mice was virtually indistinguishable from that of global α2$^{R/R}$ point-mutated mice, indicating that supraspinal α2 $GABA_AR$ were not required for antihyperalgesia induced by systemic HZ166, indicating that supraspinal α2 $GABA_AR$ did not make a significant contribution to antihyperalgesia by systemic HZ166 neither through a direct action on the nociceptive system nor through other more indirect actions such as the reversal of anxiety-induced hyperalgesia.

Fig. 5. Antihyperalgesic effects of HZ166. HZ166 was injected intraperitoneally at a dose of 16 mg/kg body weight 7 days after CCI surgery. Effects on thermal (**A, B**) and mechanical (**C, D**) hyperalgesia are expressed as percentage maximum possible analgesia (%MPE) (mean ± SEM) determined for the time interval between 1 and 2 h after drug application. ANOVA followed by Bonferroni *post hoc* test, $F = 37.55$ and 19.61 for thermal and mechanical hyperalgesia, respectively. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, significant against vehicle-treated α2$^{fl/fl}$ mice; +, $P \leq 0.05$; ++, $P \leq 0.01$; +++, $P \leq 0.001$, significant against HZ166-treated α2$^{fl/fl}$ mice.


A
Thermal hyperalgesia
%MPE
Time (h)
α2fl/fl
α2fl/fl
Hoxb8-α2-/-
Hoxb8-α2R/-
α2R/R
B
%MPE
α2fl/fl
α2fl/fl
Hoxb8-α2-/-
Hoxb8-α2R/-
α2R/R
Veh
HZ 166
C
Mechanical sensitization
%MPE
Time (h)
D
%MPE
Veh
HZ 166

This was also confirmed by additional experiments in which we investigated potential anxiolytic actions of two doses of HZ166 in the elevated plus maze test (fig. 6). Neither 8 mg nor 16 mg HZ166 /kg body weight exerted a significant anxiolytic response as indicated by the absence of a significant effect on the time spent in the open arms of the plus maze. The lack of effect on the total number of entries in this test confirmed the absence of a sedative action of HZ166 at the doses tested.

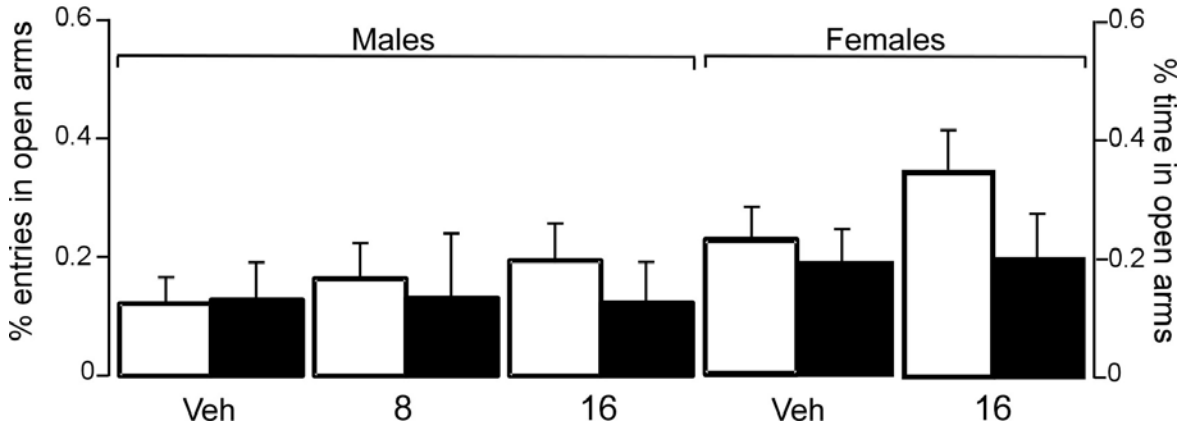


Fig. 6. Absence of anxiolytic effects of HZ166 in C57BL/6 mice in the elevated plus maze. Percentage of total entries and open arm entries in C57BL/6 mice treated with vehicle (veh) or 8 or 16 mg/kg HZ166 (8, 16 respectively. Males V-*n*=6, 8-*n*=5, 16-*n*=6 and females V-*n*=9 and 16-*n*=8. Data represent mean ± SEM. ANOVA - No significant difference was detected between the vehicle and any of the treated group.

Discussion

Studies performed in mice carrying a point mutation in the $GABA_AR$ α2 subunit gene, which renders this subunit diazepam-insensitive, have provided compelling evidence for the relevance of α2 $GABA_AR$ for diazepam-induced spinal antihyperalgesia (Knabl et al., 2008). Similar compelling evidence was lacking for more recently developed BDZ site agonists with improved subtype specificity. Our present study focused on the novel BDZ site ligand HZ166 that shows compared to diazepam not only improved subunit specificity but also reduced sedative properties. Our biochemical and electrophysiological experiments demonstrate that the H101R point mutation renders α2 $GABA_AR$ not only insensitive to diazepam but also to HZ166. The subsequent behavioral experiments, in which HZ166 was tested in point-mutated $\alpha2^{R/R}$ mice, provided for the first time direct evidence that the antihyperalgesic action of a systemically applied non-sedative agonist also occurs through α2 $GABA_AR$.

The reduced sedative properties of HZ166 and the availability of tissue-specific α2 $GABA_A$-deficient mice allowed us to address the role of the supraspinal CNS areas in BDZ-induced antihyperalgesia in the absence of sedation. This appeared to us as an important issue as α2 $GABA_AR$ are not only found in the spinal cord but also in supraspinal CNS areas where they mediate for example the anxiolytic effects of classical BDZ. It was hitherto unknown to what extent α2 $GABA_AR$ in the brain contribute to the apparent antihyperalgesic actions of BDZ site ligands.

α2 $GABA_AR$ in the brain could contribute to BDZ-induced antihyperalgesia through a genuine effect on the supraspinal nociceptive system. However, alternatively their antihyperalgesic effects could also be secondary, reflecting for example, a reversal of anxiety-induced hyperalgesia. However, the actions of HZ166 on hyperalgesia were identical in $\alpha2^{R/R}$ and *Hoxb8*-$\alpha2^{-/-}$ (and *Hoxb8*-$\alpha2^{R/-}$) mice indicating that supraspinal α2 $GABA_AR$ did have any detectable influence on HZ166-mediated antihyperalgesia. Together with our previous study (Witschi et al., 2011), in which we studied mice lacking α2 $GABA_AR$ specifically in primary nociceptors, the present data unambiguously demonstrate that the major (i.e. the α2 $GABA_AR$-mediated) component of antihyperalgesia by BDZ occurs through a genuine effect on the spinal nociceptive

system and that this antihyperalgesia is not secondary to effects of BDZ on neuronal circuits in the brain.

The data presented in this study do not completely rule out a possible contribution of $GABA_AR$ expressed outside the CNS. There is indeed evidence that $GABA_AR$ are also found on the peripheral terminals of nociceptive fibers and that exogenous activation of these receptors interferes with the excitability of these nerve fiber (Carlton et al., 1999) or with action potential propagation along these fibers (Carr et al.). However, both studies did not find evidence for an activation of these receptors by endogenous GABA, which would be a pre-requisite for a contribution of these $GABA_AR$ to pharmacological effects of BDZ. In line with this absence, experiments comparing systemically and intrathecally administered diazepam demonstrated similar degrees of antihyperalgesia (Ralvenius, Rudolph and Zeilhofer, in preparation) and hence render a contribution of peripheral $GABA_AR$ expressed outside the CNS unlikely.

An unexpected finding of our study was that the complete deletion of all spinal α2 $GABA_AR$ in *Hoxb8*-α2 $^{-/-}$ mice did not lead to changes in baseline nociceptive thresholds or in the degree of neuropathic hyperalgesia. A possible explanation might be the compensatory up-regulation of other components of inhibitory neurotransmission. Such compensatory up- or down-regulations of gene expression are in fact frequently observed in mice carrying deletions of $GABA_AR$ genes (Rudolph and Mohler, 2004). However, a relevant compensation by BDZ-sensitive $GABA_A$ can be largely ruled out on the basis of our experiments with the tissue-specific point-mutated (*Hoxb8*-α2$^{R/-}$) mice, which show unchanged levels of α2 $GABA_AR$ expression in the spinal cord. Conditional knock-out *Hoxb8*-α2 $^{-/-}$ mice and the tissue-specific point-mutated mice showed virtually identical changes in their responsiveness to HZ166.

Another surprising finding of our study was the absence of a clear anxiolytic effect of HZ166 at a dose which exerted a saturating antihyperalgesic response. This result is in clear contrast to previous findings on diazepam which showed that antihyperalgesia and sedation by diazepam occur at similar doses (Knabl et al., 2009), while anxiolysis is achieved already at much lower doses (Low et al., 2000). This is particularly surprising as the prominent contribution of α2 $GABA_AR$ to HZ166-mediated antihyperalgesia indicates that a highly level of α2 receptor activation was reached with the doses used in

our study. Although we cannot fully exclude differences in HZ166 tissue concentrations between spinal cord and brain as an underlying reason, it is possible that factors different from the nature of α subunit contained in the $GABA_AR$ complex determine differences in the susceptibility of $GABA_AR$ to different BDZ site ligands.

Acknowledgements

This work was in part supported by a grant from the Swiss National Science Foundation to HUZ (31003AB_131093). The authors thank Isabelle Camenisch, Christina Köster und Louis Scheurer for excellent technical assistance.

References

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002) PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 5:34-40.

Andre J, Zeau B, Pohl M, Cesselin F, Benoliel JJ, Becker C (2005) Involvement of cholecystokininergic systems in anxiety-induced hyperalgesia in male rats: behavioral and biochemical studies. J Neurosci 25:7896-7904.

Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87-107.

Benson JA, Low K, Keist R, Mohler H, Rudolph U (1998) Pharmacology of recombinant gamma-aminobutyric acidA receptors rendered diazepam-insensitive by point-mutated alpha-subunits. FEBS Lett 431:400-404.

Carlton SM, Zhou S, Coggeshall RE (1999) Peripheral $GABA_A$ receptors: evidence for peripheral primary afferent depolarization. Neuroscience 93:713-722.

Carr RW, Sittl R, Fleckenstein J, Grafe P GABA increases electrical excitability in a subset of human unmyelinated peripheral axons. PLoS ONE 5:e8780.

Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424:938-942.

Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ, Edwankar R, Cook JM, Zeilhofer HU (2011) HZ166, a novel $GABA_A$ receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. Neuropharmacology. 60:626-632.

Harris JA, Westbrook RF (1995) Effects of benzodiazepine microinjection into the amygdala or periaqueductal gray on the expression of conditioned fear and hypoalgesia in rats. Behav Neurosci 109:295-304.

Hosl K, Reinold H, Harvey RJ, Muller U, Narumiya S, Zeilhofer HU (2006) Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor alpha3 subunit, which mediate central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection. Pain 126:46-53.

Jasmin L, Wu MV, Ohara PT (2004) GABA puts a stop to pain. Curr Drug Targets CNS Neurol Disord 3:487-505.

Jasmin L, Rabkin SD, Granato A, Boudah A, Ohara PT (2003) Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. Nature 424:316-320.

Knabl J, Zeilhofer UB, Crestani F, Rudolph U, Zeilhofer HU (2009) Genuine antihyperalgesia by systemic diazepam revealed by experiments in $GABA_A$ receptor point-mutated mice. Pain 141:233-238.

Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy J-M, Rudolph U, Möhler H, Zeilhofer HU (2008) Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. Nature 451:330-334.

Kontinen VK, Dickenson AH (2000) Effects of midazolam in the spinal nerve ligation model of neuropathic pain in rats. Pain 85:425-431.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Luger TJ, Hayashi T, Weiss CG, Hill HF (1995) The spinal potentiating effect and the supraspinal inhibitory effect of midazolam on opioid-induced analgesia in rats. Eur J Pharmacol 275:153-162.

Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN (2006) Both alpha2 and alpha3 $GABA_A$ receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. Eur J Neurosci 23:2495-2504.

Munro G, Ahring PK, Mirza NR (2009) Developing analgesics by enhancing spinal inhibition after injury: $GABA_A$ receptor subtypes as novel targets. Trends Pharmacol Sci 30:453-459.

Munro G, Lopez-Garcia JA, Rivera-Arconada I, Erichsen HK, Nielsen EO, Larsen JS, Ahring PK, Mirza NR (2008) Comparison of the novel subtype-selective $GABA_A$ receptor-positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. J Pharmacol Exp Ther 327:969-981.

Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 60:243-260.

Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S, Roth BL, Ramerstorfer J, Furtmuller R, Sieghart W, Cook JM (2009) Antiseizure activity of novel gamma-aminobutyric acid (A) receptor subtype-selective benzodiazepine analogues in mice and rat models. J Med Chem 52:1795-1798.

Rudolph U, Mohler H (2004) Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol 44:475-498.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 401:796-800.

Sang CS, Hayes KS (2006) Anticonvulsant medications in neuropathic pain. In: Wall and Melzack's Textbook of Pain, 6th Edition (McMahon SB, Koltzenburg M, eds): Elsevier

Tatsuo MA, Salgado JV, Yokoro CM, Duarte ID, Francischi JN (1999) Midazolam-induced hyperalgesia in rats: modulation via $GABA_A$ receptors at supraspinal level. Eur J Pharmacol 370:9-15.

Wieland HA, Luddens H, Seeburg PH (1992) A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Witschi R, Johansson T, Morscher G, Scheurer L, Deschamps J, Zeilhofer HU (2010) Hoxb8-Cre mice: A tool for brain-sparing conditional gene deletion. Genesis 48:596-602.

Witschi R, Punnakkal P, Paul J, Walczak JS, Cervero F, Fritschy JM, Kuner R, Keist R, Rudolph U, Zeilhofer HU (2011) Presynaptic Alpha2-$GABA_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control. J Neurosci 31:8134-8142.

Zeilhofer HU (2008) Loss of glycinergic and GABAergic inhibition in chronic pain--contributions of inflammation and microglia. Int Immunopharmacol 8:182-187.

3.3 Selective Distribution of $GABA_A$ Receptor Subtypes in Mouse Spinal Dorsal Horn Neurons and Primary Afferents

Jolly Paul[1], Hanns Ulrich Zeilhofer[1,2], Jean-Marc Fritschy[1]

[1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland, and Neuroscience Center Zurich

[2]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology ETH Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland

Published in The Journal of Comparative Neurology (2012).

Author contributions: J.P., J.-M.F. and H.U.Z. designed research; J.P. performed research; J.P., and J.-M.F. analyzed data; J.P. and J.–M.F. wrote the paper.

Abstract

In the spinal cord dorsal horn, presynaptic $GABA_A$ receptors ($GABA_AR$) in the terminals of nociceptors as well as postsynaptic receptors in spinal neurons regulate the transmission of nociceptive and somatosensory signals from the periphery. $GABA_AR$ are heterogeneous and distinguished functionally and pharmacologically by the type of α subunit variant they contain. This heterogeneity raises the possibility that $GABA_AR$ subtypes differentially regulate specific pain modalities. Here, we characterized the subcellular distribution of $GABA_AR$ subtypes in nociceptive circuits using immunohistochemistry with subunit-specific antibodies combined with markers of primary afferents and dorsal horn neurons. Confocal laser scanning microscopy analysis revealed a distinct, partially overlapping laminar distribution of the α1-3 and α5 subunit-immunoreactivity in laminae I-V. Likewise, a layer-specific pattern was evident for their distribution among glutamatergic, GABAergic, and glycinergic neurons (detected in transgenic mice expressing vGluT2-eGFP, GAD67-eGFP, and GlyT2-eGFP, respectively). Finally, all four subunits could be detected within primary afferent terminals. C-fibers predominantly contained either α2 or α3 subunit-immunoreactivity, terminals from myelinated (Aβ/Aδ) fibers were co-labeled in roughly equal proportion with each subunit. Presence of axo-axonic GABAergic synapses was determined by co-staining with gephyrin and vesicular inhibitory amino acid transporter to label GABAergic postsynaptic densities and terminals, respectively. Co-localization of α2 or α3 subunit with these markers was observed in a subset of C-fiber synapses. Furthermore, gephyrin mRNA and protein expression was detected in dorsal root ganglia. Collectively, these results show that differential $GABA_AR$ distribution in primary afferent terminals and dorsal horn neurons allows for multiple, circuit-specific modes of regulation of nociceptive circuits.

Introduction

Chronic pain is a frequent, strongly debilitating condition that is often resistant to pharmacotherapy and therefore a major socio-economic problem. Chronic pain can result from inflammation, nerve injury, or CNS lesion. Noxious stimuli evoking pain are detected by two major classes of nociceptors, Aδ and C nociceptors, whose cell bodies are located in dorsal root ganglia (DRG) and trigeminal ganglia. The central axons of DRG-residing nociceptors terminate mainly in the superficial layers of the spinal dorsal horn (laminae I and II) (Rexed, 1952), with peptidergic and non-peptidergic C fibers being segregated in lamina II outer (IIo) and II inner (IIi), respectively (Hunt and Mantyh, 2001; Todd, 2010). The spinal cord dorsal horn processes nociceptive signals and relays them to higher brain centers, where conscious pain sensation arises. Chronic pain is a pathological condition involving altered function of primary nociceptors and of central pain circuits, manifesting in primary hyperalgesia (exaggerated pain sensations at the site of injury) and secondary hyperalgesia in uninjured healthy tissue.

In 1965, Melzack and Wall proposed the gate control theory of pain attributing a critical contribution of inhibitory interneurons (GABAergic and glycinergic) of the spinal dorsal horn in regulating nociceptive signal strength and keeping nociceptive and non-nociceptive modalities apart (Melzack and Wall, 1965). Intrathecally-applied bicuculline and strychnine, blockers of $GABA_A$ receptors ($GABA_AR$) and glycine receptors respectively, increase nociceptive reactions elicited by exposure to noxious stimuli (Roberts et al., 1986; Yaksh, 1989). Conversely, enhancing $GABA_AR$ function by spinal application of GABA or a positive allosteric modulator, such as midazolam, was shown to depress noxious stimulus-evoked activity in spinal cord neurons and to reverse neuropathic pain induced by nerve injury (Clavier et al., 1992; Eaton et al., 1999; Knabl et al., 2008; Sumida et al., 1995).

$GABA_ARs$ are ligand-gated heteropentameric ion channels assembled from a repertoire of 19 subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3). The most common subtype in the CNS is composed of two α, two β, and one γ subunit (Möhler, 2006). $GABA_AR$ containing α1, α2, α3, or α5 subunits associated with the γ2 subunit are sensitive to diazepam (Wieland et al., 1992). In the spinal cord, morphological studies revealed a considerable

heterogeneity in the distribution of $GABA_AR$ subunits. Thus, in the rat, *in situ* hybridization studies detected strong signals for $GABA_AR$ α2, α3, β3 and γ2 subunit mRNA, weak expression for α1, α5, β1, β2, γ1 and γ3, and could not detect α6 and δ subunits (Ma et al., 1993; Persohn et al., 1991; Wisden et al., 1991). Immunohistochemical analysis revealed distinct laminar distribution of major α subunit variants (Bohlhalter et al., 1996).

$GABA_AR$ can modulate spinal nociceptive processing via at least two mechanisms. Postsynaptic $GABA_AR$ in spinal cord neurons directly reduce their excitability, while $GABA_AR$ located in afferent terminals of nociceptors cause presynaptic inhibition of transmitter release. Previous work has demonstrated that the α2 and α3 subunits are abundant in mouse dorsal horn neurons (Knabl et al., 2008), co-localized in part with substance P-positive terminals in lamina II and with NK1-receptor-positive lamina I neurons. In addition, there is extensive pharmacological and behavioral evidence indicating that both modes of inhibition contribute to physiological and pathological pain sensation (Jasmin et al., 2003; Knabl et al., 2008; Munro et al., 2011; Sastry, 1980; Zeilhofer et al., 2012b). However, the precise site of action of the different $GABA_AR$ subtypes in various pain modalities is not known.

These uncertainties are due, in large part, to insufficient knowledge about the cellular and subcellular distribution of $GABA_AR$ subtypes in circuits of spinal dorsal horn (Zeilhofer et al., 2012b). Therefore, the aim of this study was to analyze immunohistochemically the molecular organization of $GABA_AR$ in identified intrinsic dorsal horn neurons and primary afferents in layers I-III, using immunofluorescence staining combined with specific neuronal and axonal markers.

Materials and Methods

Animals

Experiments were performed with 6-8 week old C57BL/6J male and female mice. For identification of glutamatergic, GABAergic and glycinergic neurons, transgenic mice expressing vGlut2-eGFP (www.gensat.org, (Gong et al., 2003), GAD67-eGFP (Tamamaki et al., 2003; Wang et al., 2009) and GlyT2-eGFP (Zeilhofer et al., 2005) on C57BL/6J background were used. Mice were bred in the animal facility of Institute of Pharmacology and Toxicology under the approval of local authorities. They were maintained under standard conditions with 12 h day/night cycle and *ad libitum* supply of food and water. All experimental procedures were approved by the Cantonal Veterinary Office of Zurich.

Western blot analysis

Mice (n=3) deeply anaesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg) were decapitated and DRG were quickly dissected out, snap-frozen with dry ice, and stored at -80°C until further processing. 30-40 DRG were homogenized in ice-cold solubilization buffer (1 mM EDTA, 10 mM Tris Cl, 150 mM NaCl, 0.02% NaN_3) followed by sonication. The protein content of the sonicated sample was determined by Bradford's assay and was then denatured using 10% mercaptoethanol. The samples were then serially diluted to achieve the required protein concentration in 20 µL loading volume. 7.5% SDS-polyacrylamide gels were used for electrophoresis and a wet-blot was developed on nitrocellulose acetate membrane. The membrane was then blocked using 5% western blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany, Cat No. 11 921 673 001) in Tris buffered saline-Tween 20 followed by overnight incubation in mouse monoclonal antibody 7a against gephyrin (Table 1). Following washing, the membrane was treated with secondary antibody goat anti-mouse conjugated to horseradish peroxidase (1:300 dilution) and the signal was enhanced using freshly prepared chemiluminescent mix (Super signal West Pico chemiluminescent substrate, Thermo, 3747N, Rockford, Cat No. 34087). The images were captured using Fujifilm scanner using the software Fujifilm-image reader LAS 100 Pro V2.51. Mouse crude brain homogenates were included in the test as positive control for gephyrin detection and albumin as a negative control.

Real-time PCR

Lumbar DRG from adult mice were removed following decapitation and collected in a tube containing a lysis solution. Total RNA was isolated by using GenElute™ Mammalian Total RNA Miniprep Kit (RTN70, Sigma-Aldrich). Genomic DNA was removed and cDNA was generated in a reaction mixture using QuantiTect® Reverse Transcription Kit (Qiagen® GmbH, Hilden, Germany, Cat. No. 205311), real-time quantitative PCR (qPCR) was performed and analyzed using TaqMan® gene expression master mix (Applied Biosystems International, Inc., Rotkreuz, Switzerland, Part No. 4369016) with generated cDNA in a total reaction volume of 10 µL in 384-well arrays with 7900HT fast real-time PCR system and software (Applied Biosystems). mRNA expression of gephyrin was quantified using pre-designed coding assays (FAM™ dye-labeled TaqMan® MGB probes; Applied Biosystems) (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cytoplasmic reference gene: assay ID: Mm99999915_g1, context sequence: GAACGGATTTGGCCGTATTGGGCGC; gephyrin: assay ID: Mm01297308_m1**, context sequence: AGAAAGGATC TCAGGAATGCTTTCA). The real-time qPCR cycling program consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression level of gephyrin gene was normalized to that of the GAPDH gene, which was used as a reference. All qPCR reactions were carried out in triplicate. Relative quantification of transcript was determined using the comparative CT method ($2^{-\Delta CT}$) calibrated to β-actin.

Immunohistochemistry

Immunohistochemistry was performed using well characterized primary antibodies described in table 1. The distribution of α subunit variants in the lumbar spinal cord was analyzed in sections processed for immunoperoxidase staining. Mice were deeply anaesthetized with pentobarbital (nembutal, 50 mg/kg, i.p.) followed by transcardiac perfusion of saline (20 mL) and 70 mL ice-cold fixative containing 4% paraformaldehyde (PFA) and 15% saturated picric acid solution in 0.15 M sodium phosphate buffer, pH 7.4. The spinal cords were extracted immediately after the perfusion and post-fixed in the same solution for 5 h. Tissue was then processed for antigen retrieval as described (Kralic et al., 2006), cryo-protected with 30% sucrose in phosphate-buffered saline (PBS), and cut coronally at 40 µm from frozen blocks with a sliding microtome. Sections were collected in PBS and stored at -20°C in antifreeze solution (15% glucose and 30% ethylene glycol in 50 mM sodium phosphate buffer, pH 7.4) prior to use. Sections were

incubated free-floating overnight at 4°C with primary antibodies against $GABA_AR$ subunits (Table 1) in Tris buffer pH 7.7 containing 2% normal goat serum and 0.2% Triton X-100. Sections were then washed and incubated for 30 min at room temperature with biotinylated secondary antibodies (1:300; Jackson ImmunoResearch, West Grove, PA), followed by incubation in avidin-biotin complex (1:100 in Tris buffer, Vectastain Elite Kit; Vector Laboratories, Burlingame, CA), for 30 min, washed again and finally visualized with diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO) in Tris buffer (pH 7.7) containing 0.015% hydrogen peroxide. The color reaction was stopped after 5–15 min with ice-cold PBS. Sections were then mounted on gelatin-coated slides and air-dried followed by dehydration with ethanol. The slides were then cleared in xylol and cover-slipped with Eukitt (Erne Chemie, Dallikon, Switzerland).

The distribution of $GABA_AR$ α subunits in identified spinal cord neurons were analyzed by immunofluorescence staining on coronal sections of transgenic eGFP-expressing mice. The sections were prepared from 2-3 male mice as described above. For staining, the sections were incubated overnight at 4°C with a mixture of primary antibodies diluted in Tris buffer containing 2% normal goat serum. Sections were washed extensively and incubated for 1h at room temperature with the corresponding secondary antibodies conjugated to Cy3 (1:500), Cy2 (1:200) (Jackson ImmunoResearch) and or Alexa488 (1:1000, Molecular Probes, Eugene, OR). Sections were washed again and cover-slipped with fluorescence mounting medium (DAKO, Carpinteria, CA).

Distribution of $GABA_AR$ α subunits within primary afferent terminals was studied using a protocol developed for detection of postsynaptic proteins (Schneider Gasser et al., 2006). Mice were deeply anaesthetized with pentobarbital (nembutal, 50 mg/kg, i.p.) and perfused with Ringer's solution. Spinal cords were rapidly collected by pressure ejection and placed in oxygenated ice-cold artificial cerebrospinal fluid. 300 µm thick parasagittal slices were prepared from the lumbar spinal cord with a vibrating microtome and incubated for 45 min at 34°C. They were then fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 10 min, extensively washed, and stored overnight in 30% sucrose in Tris buffer for cryo-protection. Slices were then flat-frozen and sections were cut at 14 µm with a cryostat, mounted on gelatin-coated glass slides and air-dried at room temperature for 45 min. Immunofluorescence staining was performed as described above.

Image acquisition and analysis

The regional distribution of α subunit-immunoreactivity (-IR) analyzed by immunoperoxidase staining was visualized in sections from four mice with bright field microscopy (Axioplan; Zeiss) using MCID Elite 6.0 software (Imaging Research, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for image acquisition. Images were cropped to the desired dimensions in Adobe Photoshop CS. Minimal adjustments of contrast and brightness were applied to entire images, if needed.

Double and triple-immunofluorescence signals were visualized by confocal microscopy (LSM 710; Zeiss AG, Jena, Germany) using a 63× Plan-Apochromat objective (N.A. 1.4). The pinhole was set to 1 Airy unit for each channel and separate color channels were acquired sequentially. The acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. Typically, stacks of confocal images (1024×1024 pixels) spaced by 0.3 µm were acquired at a magnification of 56-130 µm/pixel. For display, images were processed with the image analysis software Imaris (Bitplane; Zurich, Switzerland). Images from all channels were overlaid (maximal intensity projection) and background was subtracted, when necessary. A low-pass filter was used for images displaying α subunit staining.

Analysis of the distribution of α subunit-IR in eGFP-positive neurons and dendrites was performed in single confocal sections from 2-3 mice per genotype acquired at a magnification of 78 nm/pixel in 8-bit gray scale images, using a threshold segmentation algorithm (minimal intensity, 90–130; area >0.08 μm^2). All structures with >70% overlap between both channels (co-localized area >0.056 μm^2, corresponding to 9 pixels) was apparent were considered as "colocalized" (Image J imaging software, NIH, Bethesda, MD).

Quantification of colocalization of α subunit-IR within primary afferent terminals was likewise performed by confocal laser scanning microscopy in sections from five adult mice. The analysis was done in high magnification images acquired at the Nyquist rate (56 nm/pixel; 150 nm spacing). To minimize false-positive results, additional criteria were applied: (a) The α subunit staining is completely inside the axon terminal staining; (b) the area of colocalization >20 pixels (>0.063 μm^2); (c) co-localization was visible in

three consecutive confocal sections. The data were acquired in six sections per animal and analyzed statistically using non-parametric tests (Kruskal–Wallis followed by Dunn's multiple comparison test; Prism, GraphPad Software, San Diego, CA, USA). All files were randomized and individual terminals were identified as isolated objects and counted automatically (Imaris; Bitplane).

Target	Immunogen	Species	Source	Characterization	References
α1 subunit	Rat N-terminal synthetic peptide 1–16 (pGluPSQDE LKDNTTVFTR)	Guinea pig serum	In house	Antibody and immunohistochemistry protocol were verified in mutant mice lacking the respective subunit	(Fritschy et al., 1997; Panzanelli et al., 2011; Schneider Gasser et al., 2007; Studer et al., 2006)
α2 subunit	Rat N-terminal synthetic peptide 1–9 (NIQEDEAKN)	Guinea pig affinity purified			
α3 subunit	Rat N-terminal synthetic peptide 1–15 (pGluGESRRQEPGDFV KQ)	Guinea pig serum			
α5 subunit	Rat N-terminal synthetic peptide 1–10 (QMPTSSVQDE)	Guinea pig serum			
α4 subunit	Rat N-terminal synthetic peptide 1–14 (LNESPGQNSKDEKL)	Rabbit affinity purified	Phosphosolutions, Aurora, CO; cat. no. 844-GA4N	Antibody and immunohistochemistry protocol were verified in $\alpha4^{-/-}$ mice	(Bencsits et al., 1999; Peng et al., 2002); Fritschy, unpublished
CGRP	Synthetic calcitonin gene related peptide	Rabbit polyclonal	Chemicon, Temecula, CA; cat no. AB15360	By immunohistochemistry both antibodies react with peptidergic primary afferent terminals. Specific reactivity of these antisera is eliminated by pre-incubation with excess peptide antigens	(Chang et al., 2004; Dirmeier et al., 2008)
Substance P	Substance P conjugated to BSA	Rat monoclonal	Bachem, St.Helen, UK; cat. no. T-1609		
Cholera toxin B	cholera toxin B subunit (cholerogenoid)	Goat serum	List Biological Laboratories, Campbell, CA; cat. no. 7032A6	immunoprecipitation of cholera toxin B subunit; selective detection of CTB injected into peripheral nerves; co-localization with vGluT2 in primary afferent terminals	(Todd et al., 2003)
Gephyrin	Affinity-purified rat glycine receptors	Mouse monoclonal, clone 7a	Synaptic Systems (Gottingen, Germany); cat. no. 147011	Selective detection of gephyrin in inhibitory synapses; detection of recombinant gephyrin expressed in neurons	(Pfeiffer et al., 1984; Sassoè-Pognetto et al., 2000)
GFP	Purified recombinant green fluorescent protein	Chicken IgY fraction from yolks	Aves laboratories, Portland, OR; cat. no. GFP-1020	Western blot analysis; immunohistochemistry using transgenic mice expression the GFP gene product.	(Encinas et al., 2006)

Protein kinase Cγ	Synthetic peptide 676-689 of human PKCγ (VNPDFVHPDARSPT)	Mouse monoclonal, clone 20	BD Transduction laboratories; cat no. 611158	Western blot analysis; immunohistochemistry	(Neumann et al., 2008)
Versican	recombinant protein fragment of GAG-α domain of mouse versican	Rabbit affinity purified	gift from Prof. Dieter Zimmermann	Immunoblotting of the fusion protein and crude mouse brain extracts; co-localization with IB4 in primary afferent terminals	(Schmalfeldt et al., 1998; Zimmermann et al., 1994)
VIAAT	Synthetic peptide 75-87 of rat VIAAT (AEPPVEGDIHYQR)	Rabbit affinity purified	Synaptic Systems; cat. no. 131003	Specific for the mammalian vGAT demonstrated by Western blotting and immunohistochemistry; selective detection of GABAergic terminals in brain sections and primary neuron cultures	(Brünig et al., 2002; Fritschy et al., 2006; Panzanelli et al., 2009)
vGluT1	Recombinant GST-fusion protein containing amino acid residues 456-561 of rat vGluT1	Rabbit serum	Synaptic Systems; cat. no. 135002	Specific for the mammalian vGluT1 demonstrated by Western blotting and immunohistochemistry	(Bellocchio et al., 2000; Fujiyama et al., 2001; Todd et al., 2003)
vGluT2	Recombinant GST-fusion protein containing amino acid residues 510-582of rat vGluT2	Rabbit serum	Synaptic Systems; cat. no. 135102	Specific for the mammalian vGluT2 demonstrated by Western blotting and immunohistochemistry; selective detection of a subset of glutamatergic axon terminals in brain sections	(Fremeau et al., 2004; Nahmani and Erisir, 2005)

The nonpeptidergic fibers were identified by the marker Isolectin B4-alexa 488 conjugate from Molecular probes, Eugene, OR, USA, cat no. 121411.

Tab. 1. Primary antibodies used.

Results

Differential distribution of $GABA_AR$ α subunits in mouse spinal dorsal horn

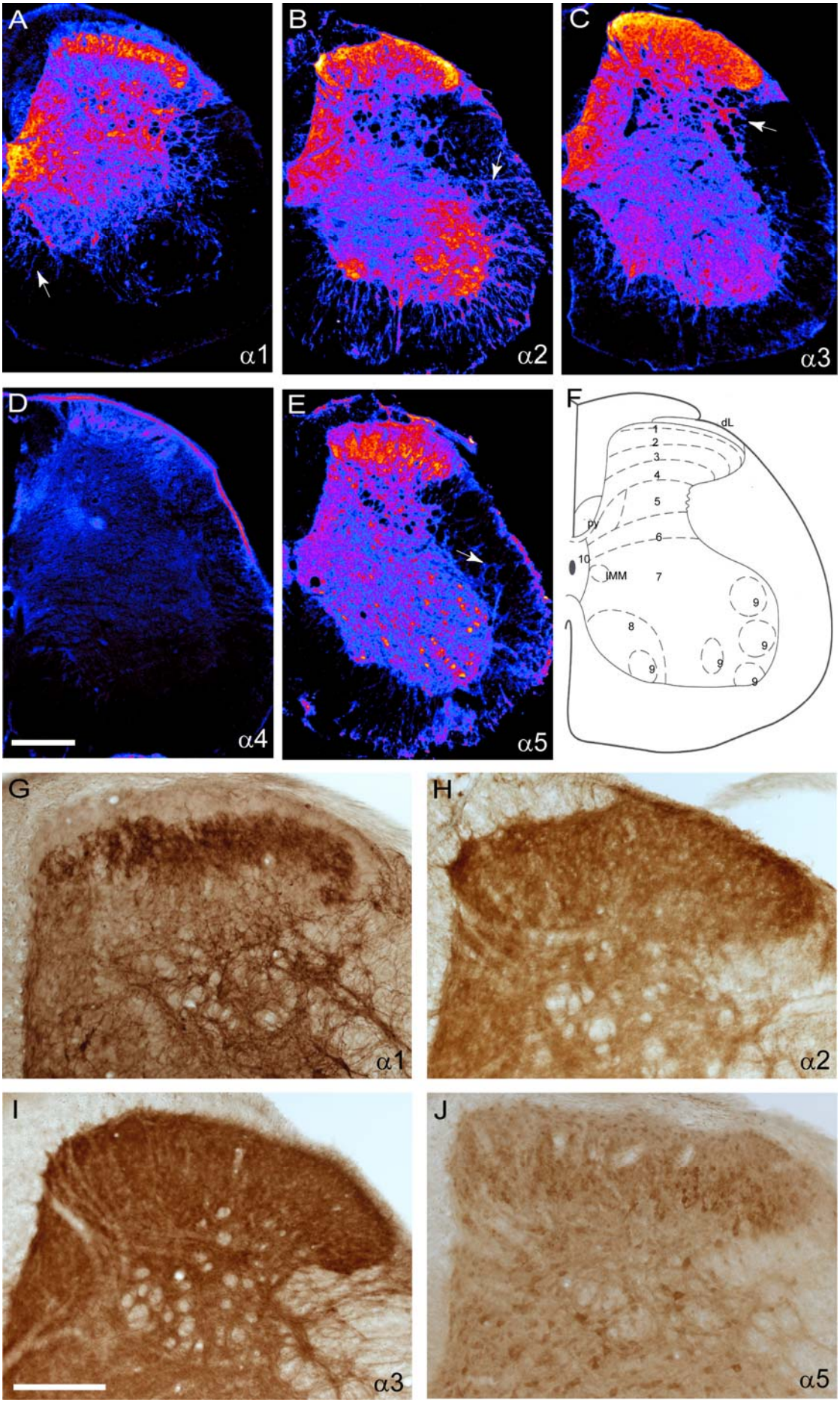
The distribution of α subunit variants was examined in transverse sections of the lumbar spinal cord using immunoperoxidase staining with subunit-specific antibodies. In order to visually enhance the differences in staining intensity across layers, images are displayed in false-colors ranging from dark blue for weak signals to red, pink, orange, yellow and white for maximal intensity (Fig. 1A-E). For each antibody, signals were normalized to display the strongest signals in white, except for the α4 subunit, where only background was detected. Higher magnification images of the dorsal horn are also shown in color photomicrographs of immunoperoxidase staining (Fig. 1F-I). At the macroscopic level, α1, α2, α3, and α5 subunit immunoreactivity showed a widespread but differential laminar distribution pattern, whereas the α4 subunit was not detectable although a strong signal was observed in specific forebrain regions (e.g., thalamus) using the same staining conditions (not shown). As described previously in rat (Bohlhalter et al., 1996), IR was restricted to the grey matter except for dendrites extending radially into the white matter. In lamina I, mainly the α2 and α3 subunits were detected, along with a few α1 subunit-positive cells. Staining for the α2 and α3 subunits was most intense in lamina II, along with a moderate α5 subunit-IR. In addition, the α1 subunit was detected in the innermost lamina II. In lamina III, all four subunit variants were present with a peak staining intensity for the α3 and α5 subunit, a moderate α2 subunit staining and weak α1 subunit-IR. The latter was stronger in laminae IV-V in which α2, α3, and α5 subunit-IR was also detected at moderate levels. A distinct gradient of intensity was evident medio-laterally, culminating with intense staining of lamina X, around the central canal, notably for the α1, α2, and α3 subunit. In summary, a roughly complementary distribution pattern was observed between the α1 and α2 subunit in laminae I-III, whereas the α3 and α5 subunit largely overlapped in lamina III. The overall strong IR of these four α subunits (which all contribute to assembly of diazepam-sensitive $GABA_AR$) in the superficial dorsal horn compared with the intermediate zone and ventral horn underscores the relevance of $GABA_AR$ for processing of nociceptive inputs in the spinal cord.

Neuron-specific expression of α subunit variants

To determine whether these differential distribution patterns reflect possible neural circuit specificity, we investigated the distribution of the four α subunit variants in neurons identified by their transmitter phenotype (glutamatergic, GABAergic, glycinergic) using transgenic mice expressing vGluT2-eGFP, GlyT2-eGFP, and GAD67-eGFP, respectively. To unambiguously determine the laminar distribution of these neuronal populations, CGRP was used as a marker to differentiate lamina I from lamina IIo (Fig. 2A) and IB4 for separating lamina IIo from lamina IIi. In preliminary experiments, we confirmed that IB4 labeling is limited to the outer part of lamina IIi (Fig. 2A), using PKCγ as a specific marker of lamina II-III boundary (Neumann et al., 2008). Figure 2B-C illustrates in fluorescence microscopy the three classes of interneurons with respect to IB4 labeling. Both vGluT2-eGFP- and GAD67-eGFP-positive neurons were distributed evenly in laminae IIo, IIi and III, whereas GlyT2-eGFP-positive neurons were mainly located in laminae III-IV and scarcely in lamina IIo at the border with lamina I.

We analyzed systematically each neuronal population to determine which proportion of them was immunopositive for the α2/α3 subunits (most abundant in layer II) and α1/α5 subunits (most abundant in layers III-IV). Data are pooled from 2-3 mice per genotype, with ≈50 randomly selected eGFP-positive neurons per mouse being assessed in each lamina for colocalization with α subunit-IR. This analysis was performed in perfusion-fixed tissue, in which eGFP signals are optimally preserved, notably in thin dendritic profiles. Under these conditions, staining was rather uniform and diffuse in the neuropil and it was not possible to distinguish postsynaptic from extrasynaptic $GABA_AR$ subunit staining (Fritschy et al., 1998). Double-labeled dendrites were also seen, but were not quantified, as they could not be allocated to identified cells. Therefore, quantitative analysis was restricted to double-labeled cell bodies.

Fig. 1. Differential distribution of $GABA_AR$ α subunit variants in adult mouse lumbar spinal cord. **A-E:** Each panel shows a color-coded photomicrograph of a coronal hemi-section processed for immunoperoxidase staining with subunit specific antibodies. The intensity of pixel was normalized using a custom-made look-up table showing the strongest signal in white and the background in dark blue, except for the α4 subunit, where only background staining was detected. Comparisons between antibodies are not relevant. Immunoreactive dendrites penetrating into the white matter are indicated with arrows. Notice the intense α2- **(B)** and α3 subunit **(C)** IR in lamina I and II, and α1 **(A)**, α3 and α5 subunit-IR **(E)** in lamina III-V, whereas α4 subunit-IR **(D)** was not detectable. In addition, the α1, α2, and α3 subunit-IR was prominent around the central canal (lamina X). **F:** Schematic representation of spinal cord laminae**. G-J**: Color photomicrographs of the dorsal horn, depicting the cellular distribution of the α1, α2, α3, and α5 subunit in the superficial laminae. Scale bars, 100 μm.

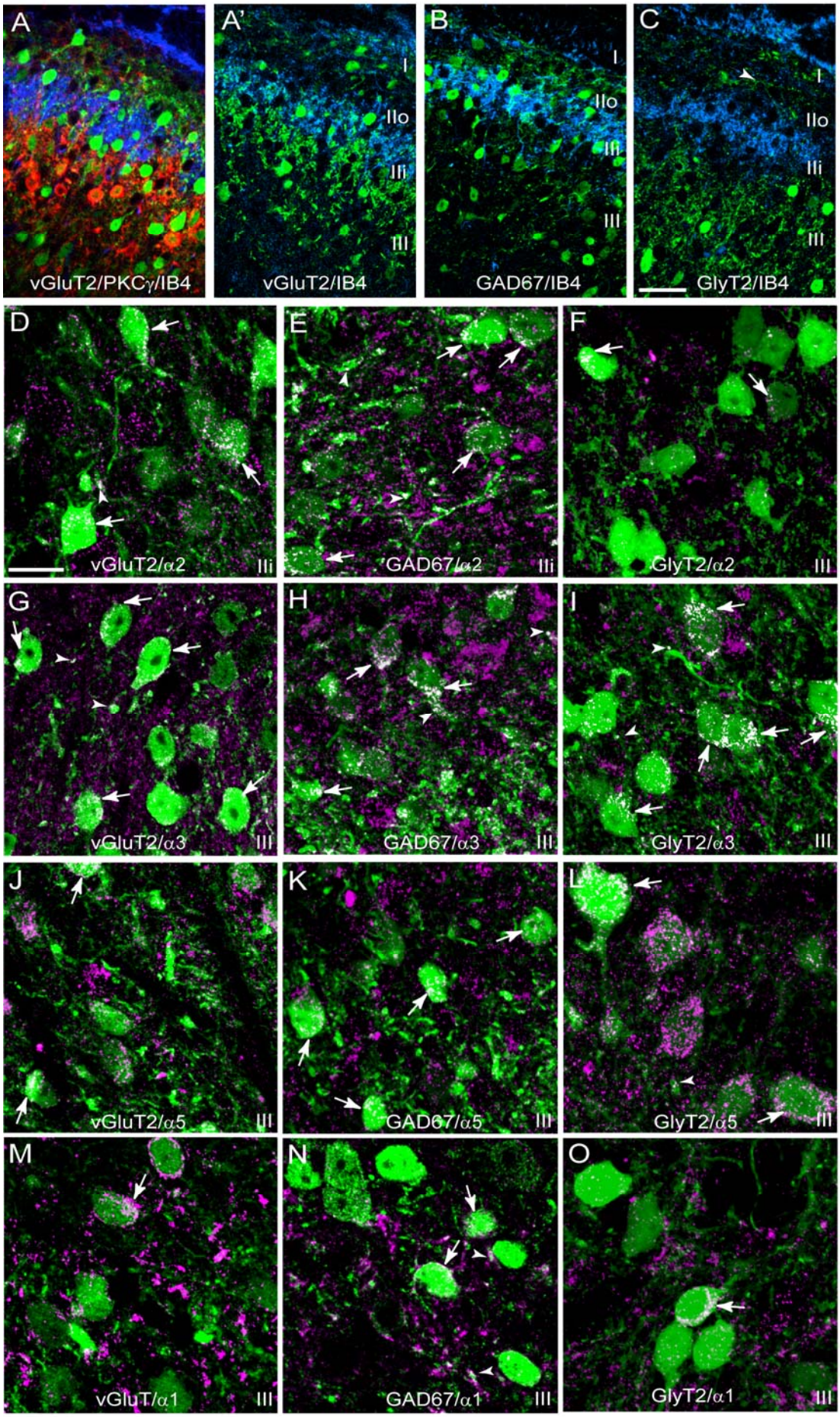

A
α1
B
α2
C
α3
D
α4
E
α5
F
dL
1
2
3
4
5
py
6
10
IMM
7
8
9
9
9
9
9
G
α1
H
α2
I
α3
J
α5

Further, we could not determine whether multiple α subunit variants were present in the same cell; however, this information is inferred when the total percentage of colocalization exceeds 100%. Representative examples of each colocalization pattern are shown in Figure 2D-O and the quantitative results are provided in Figure 3A-C.

vGluT2-eGFP-positive glutamatergic neurons

Considerable laminar specificity was observed in the distribution of α subunit variants in vGluT2-eGFP-positive cells, with α2/α3 subunit-IR being each present in about 40% of cells in lamina IIo, along with the α5 subunit in a small fraction of cells, whereas in lamina IIi, almost all eGFP-positive cells were double-labeled for the α3 subunit and >50% for the α2 subunit. In lamina III, vGluT2-eGFP-positive cells likely expressed more than one α subunit variant, the proportion of co-localization ranging from 25% for the α2 subunit to 70% for the α3 subunit, with a significant contribution of α1 and α5 subunit, as well (Fig. 3A). These percentages of colocalization well matched the fraction of dendritic profiles immunoreactive for each subunit in the various laminae (Fig. 2D, G, J, M), suggesting overall a somato-dendritic localization of $GABA_AR$ in glutamatergic dorsal horn interneurons and a major influence of α3-$GABA_AR$, notably in lamina IIi.

Fig. 2. Layer-specific allocation of α subunit variants in identified subpopulations of eGFP-positive dorsal horn neurons. **A-C:** Images depicting vGluT2-eGFP (A-A'), GAD67-eGFP (**B**) and GlyT2-eGFP (**C**) neurons (green) in laminae I-III distinguished by IB4 (blue) labeling of non-peptidergic primary afferents, as well as PKCγ (red, panel A only). **D-O**: Double fluorescence labeling with antibodies to the α1, α2, α3, and α5 subunit (magenta) and eGFP (green), depicting colocalized voxels in white. Each image represents a stack of 13 confocal layers spaced by 0.3 μm. Each column represents a different neuronal marker; the lamina from which each image was taken is indicated in the lower right corner; neurons identified as double-labeled are shown with arrows, whereas arrowheads point to examples of double-labeled dendrites. Note that the majority of α subunit immunoreactive puncta are not associated with eGFP-positive dendrites; even in those cases when most somata are double-labeled (e.g., panels E or H). Scale bars: A-C 20 μm; D-O 10 μm.


A
vGluT2/PKCγ/IB4
A'
I
IIo
IIi
III
vGluT2/IB4
B
I
IIo
IIi
III
GAD67/IB4
C
I
IIo
IIi
III
GlyT2/IB4
D
vGluT2/α2
IIi
E
GAD67/α2
IIi
F
GlyT2/α2
III
G
vGluT2/α3
III
H
GAD67/α3
III
I
GlyT2/α3
III
J
vGluT2/α5
III
K
GAD67/α5
III
L
GlyT2/α5
III
M
vGluT/α1
III
N
GAD67/α1
III
O
GlyT2/α1
III

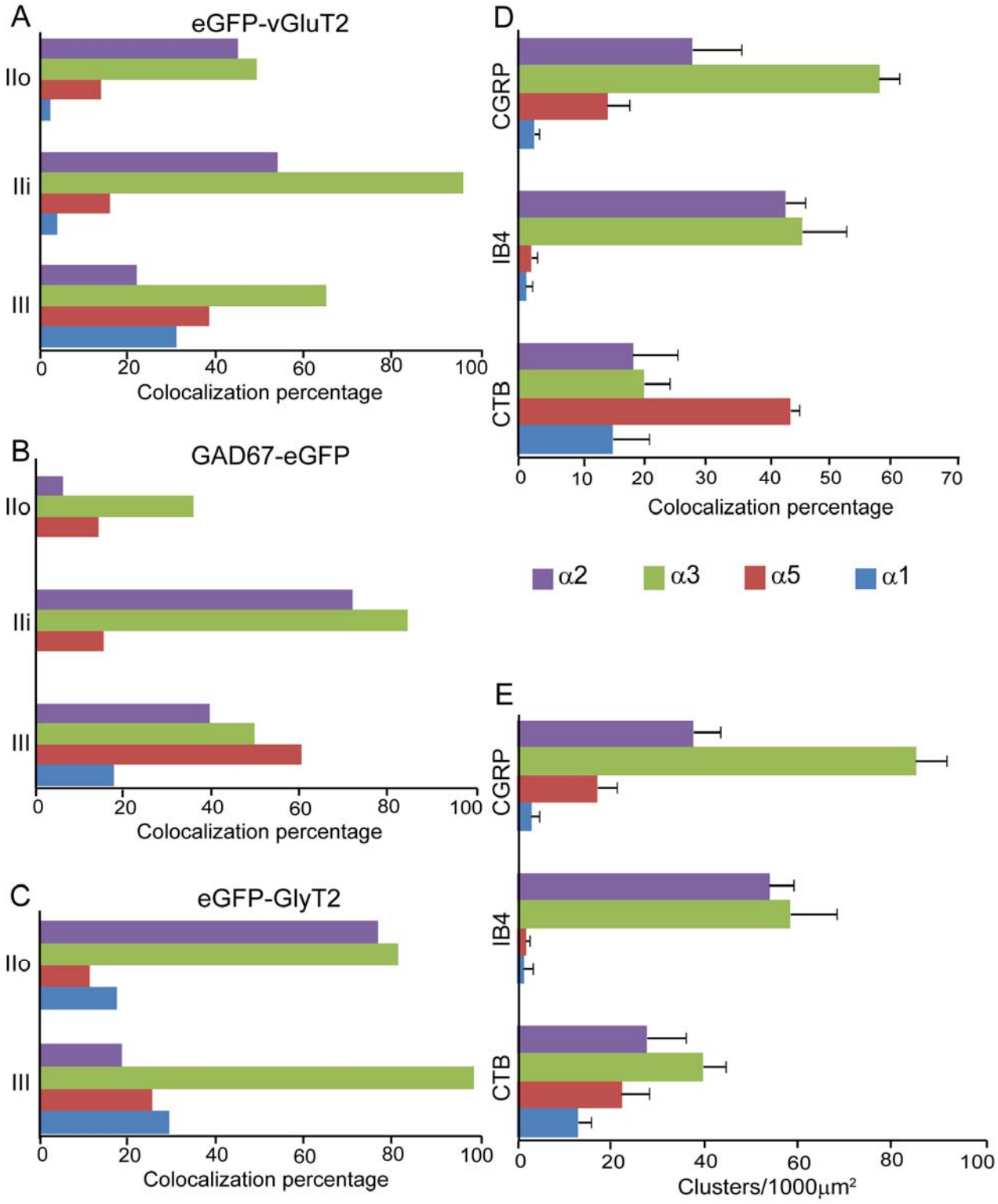
GAD67-eGFP-positive GABAergic interneurons

In contrast to the pattern shown by excitatory interneurons, only around 5% GAD67-eGFP-positive cells in lamina IIo had α2 subunit-IR, along with 72% of cells in lamina IIi. Besides, α2 subunit-IR was present in around 40% cells located at the lamina IIi-III border. α3 subunit-IR was observed in 37 and 85% of GAD67-eGFP-positive cells in lamina IIo and IIi respectively. Therefore, in lamina IIi most of these cells have both α2- and α3-$GABA_AR$. In lamina III, α3 subunit-IR was observed in 50% of GAD67-eGFP-positive cells and the α5 subunit-IR in 60% of them, suggesting possible co-occurrence of these subunits in individual neurons. As seen for vGluT2-eGFP neurons, a large fraction of GAD67-eGFP-positive dendritic profiles were double-labeled for the α2 or α3 subunits in these experiments (Fig. 2E, H, K, N), suggesting that GABAergic synapses are distributed onto both dendritic and perisomatic areas.

GlyT2-eGFP-positive glycinergic neurons

GlyT2-eGFP-positive glycinergic cell somata were strikingly rare lamina II (absent in lamina IIi) and were more frequently detected in laminae III-V. The majority of the few cells in lamina IIo contained both α2 and α3 subunits and almost all GlyT2-eGFP-positive neurons in deeper laminae were positive for the α3 subunit-IR. The α1 and α5 subunits were detected in a few fluorescent cells of lamina IIo, (around 10-15%) and about 25% in lamina III (Fig. 3C). The dendrites of glycinergic neurons were only occasionally labeled for a $GABA_AR$ subunit (Fig. 2F, I, L, O), suggesting a concentration of inputs in the perisomatic area.

Fig. 3. Quantitative analysis of the distribution of α subunit variants in dorsal horn eGFP-positive neurons and in primary afferent terminals. **A-C**: Colocalization frequency of α subunit IR in vGluT2-eGFP **(A)**, GAD67-GFP **(B)** and GlyT2-eGFP **(C)** neurons, subdivided by lamina. The data are pooled from 2-3 mice per genotype. **D**: Percentage colocalization (mean ± SD) of CGRP- (lamina IIo), IB4- (lamina IIi), and CTB- (lamina III) positive primary afferent profiles with α subunit variants in C57BL/6J mice. **E**: Density of α subunit immunoreactive clusters (mean ± SD; n=3 mice/group) colocalized with CGRP-, IB4-, and CTB-positive profiles. Colocalization was quantified in six sections per mouse.

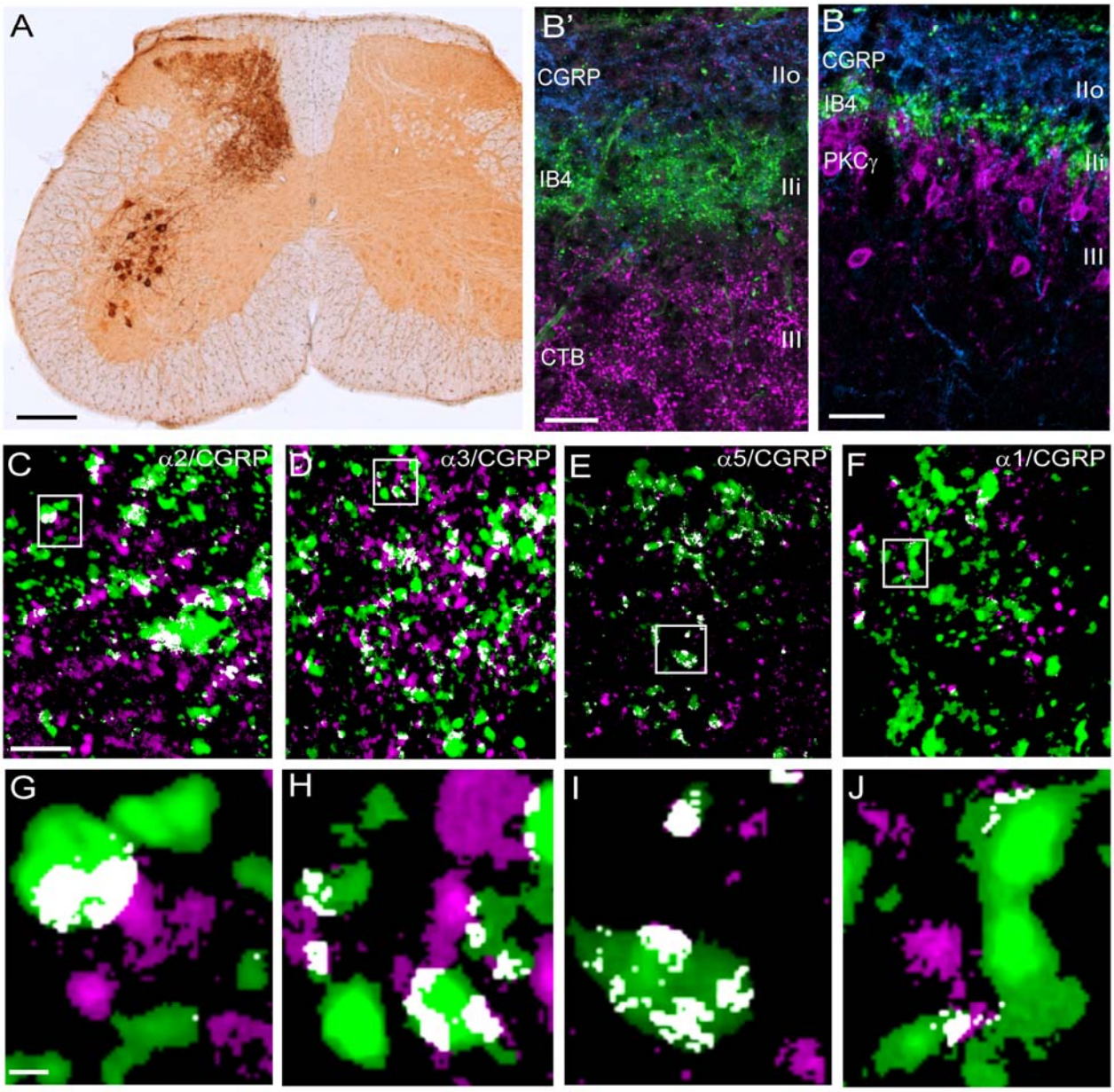

A
eGFP-vGluT2
IIo
IIi
III
0 20 40 60 80 100
Colocalization percentage
B
GAD67-eGFP
IIo
IIi
III
0 20 40 60 80 100
Colocalization percentage
C
eGFP-GlyT2
IIo
III
0 20 40 60 80 100
Colocalization percentage
D
CGRP
IB4
CTB
0 10 20 30 40 50 60 70
Colocalization percentage
α2 α3 α5 α1
E
CGRP
IB4
CTB
0 20 40 60 80 100
Clusters/1000μm²

$GABA_AR$ subunit distribution in primary afferent terminals

A large fraction of the neuropil staining seen for the various α subunit variants was not colocalized with dendritic profiles. Therefore, we investigated whether this pattern reflects the presence of $GABA_AR$ in primary afferent fiber terminals. CGRP and IB4 labeling were used to reveal peptidergic and non-peptidergic C-fibers, respectively. The specificity of these markers was confirmed by double labeling with antibodies against substance P to label peptidergic fibers and against the extracellular matrix proteoglycan versican 2, an identified IB4-binding glycoconjugate (Bogen et al., 2005; Gibbins et al., 1987) (data not shown). Myelinated fibers in the dorsal horn were labeled by transganglionic tracing of cholera toxin subunit B (CTB) injected into the sciatic nerve (Fig. 4A), as well as by immunofluorescence against vGluT1 (data not shown) (Todd et al., 2003). Triple labeling experiments performed using antibodies against CGRP and CTB along with either anti-PKCγ or fluorescently-tagged IB4 to confirm the distinct laminar distribution of these markers in primary afferent terminals in the dorsal horn (Fig. 4B-B'). CGRP, IB4 and CTB were then analyzed separately for colocalization of α subunit-immunofluorescence.

For all α subunit variants, we observed at high magnification that the punctate staining of the neuropil was partially colocalized with each of the three markers of primary afferents. For quantification, colocalization was defined by an intensity threshold algorithm using stringent criteria (see Materials and Methods). It is of note, however, that the region containing the presynaptic active zone could not be identified in primary afferent profiles, because neither vGluT1 nor vGluT2 are detectable in the terminals of C-fibers. Quantitative results are presented in Fig. 3D-E, representing average data (± SD) from 5 mice per group.

Fig. 4. Immunohistochemical identification of α subunit-IR inside primary afferent terminals. **A:** Illustration of transganglionic and retrograde transport of CTB injected into the sciatic nerve. Note the extensive labeling of axons in the ipsilateral dorsal horn and neuronal somata in the ventral horn. **B:** Lamina-specific distribution of the three classes of primary afferents in the superficial dorsal horn, demonstrated by triple labeling for CGRP (peptidergic fibers; blue) in lamina IIo, IB4 (non-peptidergic fibers; green) in lamina Iii, and CTB-positive myelinated fiber terminals (magenta) in lamina III. **B'**: Triple labeling for CGRP (green), IB4 (blue) and PKCγ (magenta) confirmed the localization of non-peptidergic afferents in the outer part of lamina IIi. **C-F:** Representative images for double staining experiments depicting with high resolution the punctate distribution of α subunit IR (magenta) relative to afferent terminals (green), as well as their colocalization (white). Framed areas are enlarged in panels **G-J**. Scale, A, 200 μm; B: 20 μm; C-F: 5 μm; G-I: 0.5 μm.

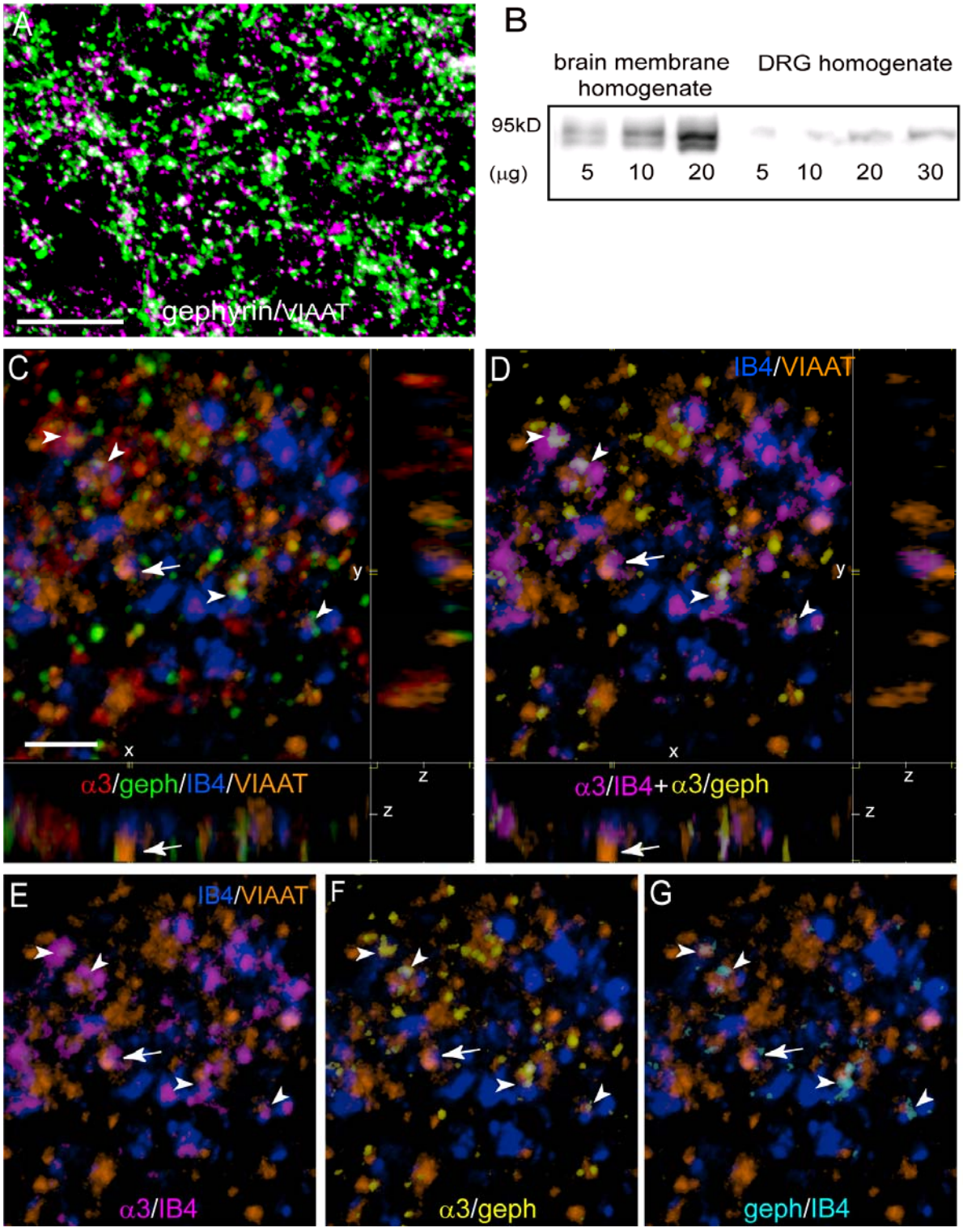

A
B'
CGRP
IIo
IB4
IIi
III
CTB
B
CGRP
IB4
IIo
PKCγ
IIi
III
C
α2/CGRP
D
α3/CGRP
E
α5/CGRP
F
α1/CGRP
G
H
I
J

In lamina IIo, a large fraction of CGRP-positive profiles, 28% and 59%, respectively, contained α2 and α3 subunit-IR (Fig. 4A-J). Likewise, 43% and 46% of IB4-labeled profiles in lamina IIi, and 18% and 21% of CTB-positive profiles in lamina III were colocalized with α2 and α3 subunit-IR (Fig. 3D). The density of puncta immunoreactive for each subunit was also analyzed (Fig. 3E). Lamina IIo showed a density of 38 and 85 α2 and α3 subunit puncta, respectively, colocalized with CGRP-IR per 1000 μm^2. In lamina IIi and III, 56 α2 subunit and 58 α3 subunit puncta were co-localized with IB4 labeling, and 28 and 40 with CTB-IR.

The α1 subunit was scarcely detected in CGRP- and IB4-positive profiles in lamina II, suggesting that it is not associated with C-fibers (Fig. 3D, E), in spite of the moderate staining intensity for this subunit in lamina IIi (Fig. 1). In contrast, α1 subunit-IR was detected in 13% CTB-positive profiles in lamina III, representing 15 puncta per 1000 μm^2 (Fig. 3E).

The α5 subunit showed high colocalization rate with CGRP-positive profiles (15%), but less than 3% with IB4-labeled profiles (Fig. 3D). The highest colocalization rate for the α5 subunit was detected in CTB-IR profiles, reaching 44%. The density analysis showed 17, 1 and 22 puncta per 1000 μm^2 in CGRP, IB4 and CTB staining, respectively (Fig. 3E).

Fig. 5. Evidence for axo-axonic GABAergic synapses on primary afferent terminals. **A:** Postsynaptic distribution of gephyrin clusters (green) in the mouse spinal dorsal horn demonstrated by extensive apposition to presynaptic terminals positive for VIAAT (blue; arrow). **B:** Western blot analysis of gephyrin in crude mouse brain extracts and dorsal root ganglia. All bands, loaded with three different protein amounts, are from a single blot. C-G: Representative image showing the postsynaptic localization of α3 subunit and gephyrin clusters in primary afferent terminals apposed to a VIAAT-positive terminal. Quadruple labeling was performed in fresh-frozen tissue to optimize detection of postsynaptic proteins. C: Quadruple staining for the α3 subunit (red), gephyrin (green), IB4 (blue) and VIAAT (orange); the stack of confocal images is displayed in the 3 Cartesian planes, with arrowheads pointing to axo-axonic synapses and the arrow to a terminal containing the α3 subunit, but not gephyrin, shown in 3D. **D:** Distribution of pixels depicting α3 subunit/gephyrin (yellow) and α3 subunit-IB4 (magenta) co-localization relative to primary afferents and VIAAT-positive terminals, showing quadruple labeling of axo-axonic synapses (white; arrowheads). **E-G**: Patterns of colocalization between pairs of markers depicted in color-separated panels, as indicated. Scale, A: 5 μm; C-G: 3 μm.


A
gephyrin/VIAAT
B
brain membrane homogenate
DRG homogenate
95kD
(µg)
5
10
20
5
10
20
30
C
α3/geph/IB4/VIAAT
x
y
z
z
D
IB4/VIAAT
α3/IB4+α3/geph
x
y
z
z
E
IB4/VIAAT
α3/IB4
F
α3/geph
G
geph/IB4

These data reveal a clear specificity in the occurrence of $GABA_AR$ subtype markers in primary afferent terminals of the superficial dorsal horn, with peptidergic and non-peptidergic fibers being associated mainly with the α3 and α2 subunits; in addition, some peptidergic fibers contain α5-$GABA_AR$, whereas CTB-positive profiles, representing myelinated fiber terminals, are associated with the four diazepam-sensitive α subunit variants.

Identification of putative GABAergic synapses on primary afferent terminals

The presence of $GABA_AR$ in primary afferent suggests a major role for filtering incoming nociceptive sensory information. To determine whether this function is assumed by GABAergic synapses, we carried our analysis further to visualize postsynaptic α2 and α3 subunit clusters, apposed to presumptive presynaptic terminals identified by staining for vesicular inhibitory transporter (VIAAT) and colocalized with gephyrin within labeled primary afferent terminals. These two markers allow for a selective discrimination of pre- and postsynaptic components of inhibitory synapses. These experiments were performed in weakly fixed tissue to maximize the detection of postsynaptic proteins (see Materials and Methods). As a prerequisite, double immunofluorescence experiments showed a systematic apposition of VIAAT-positive profile to gephyrin clusters, indicating the presence of postsynaptic $GABA_AR$ (Fig. 5A). While a large fraction of these gephyrin clusters likely are located postsynaptically in dendrites and neuronal somata, confocal laser scanning microscopy analysis showed gephyrin clusters also in a subset of peptidergic and non-peptidergic C-fibers in laminae I-II, as well as myelinated fibers in laminae III-V (data not shown). A substantial fraction (≈30%) of these gephyrin clusters was colocalized with the α2 and α3 subunit-IR.

Quadruple immunohistochemistry was performed eventually with the combination of VIAAT, gephyrin, a primary afferent marker and α2 or α3 subunit antibody (Fig. 5C-G), using primary antibodies raised in different species. We detected gephyrin along with either α2 or α3 subunit-IR in individual primary afferent terminal profiles from all three classes apposed to a VIAAT-profile, suggestive of a synaptic contact. A representative example is shown for the α3 subunit in IB4-positive terminals (Fig. 5C-G). These observations provide a proof-of-principle for the presence of α2- and α3-$GABA_AR$ associated with gephyrin in axo-axonic synapses onto primary afferent terminals.

Gephyrin mRNA and protein expression in DRG neurons

To validate the presence of gephyrin-IR inside primary afferent terminals, we verified that gephyrin is expressed in the DRG using qRT-PCR and Western blotting. Thus, qPCR analysis using Taqman assays using β-actin as reference confirmed the detection of gephyrin mRNA in DRG lysate. Relative to GAPDH, gephyrin mRNA copy number detected was 2.25±1.1%. Likewise, Western blot analysis of gephyrin in crude extracts of lumbar DRG and adult mouse brain homogenate as control confirmed the presence of gephyrin protein in both preparations, detected as a band with apparent molecular weight of 95 kDa (Fig. 5B).

Discussion

The present results demonstrate that the differential laminar distribution of $GABA_AR$ subtypes in the dorsal horn, distinguished by their α subunit variant, corresponds to a complex cell-specific and primary afferent-specific expression pattern. These findings provide a framework to investigate how $GABA_AR$ subtypes differentially regulate sensory processing and pain by controlling the activity of different neuronal networks. The highest degree of $GABA_AR$ heterogeneity occurs in lamina III, suggesting the existence with multiple parallel circuits controlled by distinct $GABA_AR$, as well as convergence of circuits impinging onto spinal cord neurons that express multiple $GABA_AR$ subtypes. $GABA_AR$ in glutamatergic primary afferents and neurons likely mediate inhibition by controlling transmitter release and postsynaptic excitation, respectively. In contrast, $GABA_A$-mediated inhibition of GABAergic interneurons might be of particular relevance for disinhibition of specific neuronal circuits (Labrakakis et al., 2009). Pharmacologically, $GABA_AR$ heterogeneity in spinal cord circuits is of immediate relevance for the treatment of distinct pain modalities with subtype-selective ligands.

Methodological considerations

Compelling evidence from morphological, functional, and pharmacological studies indicates that the six α subunit variants correspond to distinct $GABA_AR$ subtypes (Fritschy and Brunig, 2003; Olsen and Sieghart, 2008; Rudolph et al., 2001; Sieghart, 2006) and, therefore, can be used as subtype markers for immunohistochemistry. Importantly, because an α subunit is required for assembly and cell surface expression of most $GABA_AR$ (Kralic et al., 2006; Panzanelli et al., 2011; Studer et al., 2006; Sur et al., 2001; Vicini et al., 2001), their detection by immunofluorescence implies the presence of β and γ subunit variants in the same cells. However, one cannot distinguish in neurons expressing two different α subunit variants whether they are part of the same receptor complex or correspond to two receptor subtypes intermingled within the same synapse (Panzanelli et al., 2011). Therefore, it is not possible to define morphologically or functionally all neuronal circuits controlled by a given $GABA_AR$ subtype.

Despite the importance of inhibitory neurotransmission for nociception, there is only limited information available on the cellular and subcellular distribution of $GABA_AR$ in the spinal cord dorsal horn, in part due to technical limitations. First, the small size of

neurons and thin diameter of their dendrites, combined with their high packing density and the presence of numerous immunopositive primary afferent terminals, makes it very difficult to assign an immunopositive profile to an identified cell. In particular, the distinction between postsynaptic and extrasynaptic $GABA_AR$, which in brain sections can be achieved in mildly-fixed tissue (Schneider Gasser et al., 2006), is obscured in the spinal cord by the extensive labeling of primary afferent terminals. Furthermore, using transgenic mouse lines, which express eGFP in a neurotransmitter-specific manner, it is not certain that all neurons using this neurotransmitter are labeled. In particular, GABAergic neurons expressing only GAD65-only are not visible in GAD67-eGFP mice (Tamamaki et al., 2003; Wang et al., 2009). Likewise, while vGluT2-eGFP is expressed selectively in glutamatergic neurons, it is not known which fraction of these cells express this marker. A further limitation of the method is that it did allow us to quantify double labeled cells only on the basis of somatic, but not dendritic labeling.

With regard to primary afferent terminals, colocalization analysis is limited by the resolution of confocal microscopy, in particular along the z-axis. We have taken a conservative approach based on stringent criteria for assessing the presence of α subunit-IR inside labeled primary afferent terminals (see Materials and Methods). The validity of this approach was confirmed recently by the analysis of the α2 subunit in mice carrying a conditional deletion of *Gabra2* selectively in nociceptors (Witschi et al., 2011). However, in the absence of a marker of the presynaptic active zone or presynaptic vesicles in primary afferent terminals, it was not possible to determine the localization of α subunit-IR relative to these structures. To overcome this problem, we attempted to determine the presence of axo-axonic GABAergic synapses onto primary afferent terminals, using VIAAT and gephyrin as markers. This approach provided strong evidence that such synapses exist in principle, in line with ultrastructural evidence for the presence of axo-axonic GABAergic synapses on primary afferent glomeruli (Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva et al., 1985; Todd, 1996). However, although our approach did not allow a reliable quantification of their abundance, only a minority of profiles immunopositive for CGRP or IB4, as well as those labeled with CTB appeared to contain a gephyrin cluster. Such negative results may not be fully conclusive, especially when presynaptic terminals cannot be distinguished from preterminal axons, but they still suggest that in the majority of cases, $GABA_AR$ in C-fiber

terminals or in myelinated nociceptor terminals are activated by GABA spillover in the extracellular space, rather than by phasic GABA release.

Organization of $GABA_AR$ subtypes in intrinsic spinal dorsal horn neurons

Despite these limitations, our results clearly show that the four α subunit variants contributing to the assembly of diazepam-sensitive $GABA_AR$ (Rudolph and Mohler, 2006) are present in the spinal cord dorsal horn with distinct laminar, cellular and subcellular distributions. Therefore, modulation of distinct pain modalities and/or functional alterations of GABAergic inhibition in specific neuronal circuits in the dorsal horn can be expected from our results in models of chronic pain. Moreover, the failure to detect the α4 subunit indicates that this diazepam-insensitive $GABA_AR$ subtypes likely plays only a minor role in the spinal cord.

We observed a distinct laminar distribution of the α1, α2, α3, and α5 subunit-IR with considerable overlap within individual laminae. These findings imply that some neurons express multiple α subunit variants (notably α2/α3 in lamina IIo), whereas others have a single α subunit (notably in lamina IIi and III). A striking overall feature is the prominent α2 subunit staining in lamina II – also observed in the rat (Bohlhalter et al., 1996), which contrasts with the lack of α2 subunit mRNA expression reported in the dorsal horn (Persohn et al., 1991; 1992; Wisden et al., 1991). However, our present data demonstrate the presence of eGFP-positive cells immunoreactive for the α2 and α3 subunit in lamina II, with a predominance of the α3 subunit-IR (Knabl et al., 2008). Therefore, in situ hybridization with radiolabeled oligonucleotides might lack sensitivity for detecting α2 subunit mRNA in lamina II neurons. Nevertheless, most α2 subunit-IR was located in the neuropil, presumably on primary afferent terminals and possibly also on dendrites from neurons located in deeper layers.

Glutamatergic neurons identified by vGluT2-eGFP expression likely express different α subunits in lamina IIo (either α2 or α3 subunit) and III (α1, α5, α3), and both α2/α3 subunits in lamina IIi, providing a clear example of differential control by distinct $GABA_AR$ subtypes. The implications of such observations are that subtype-selective $GABA_AR$ ligands would produce distinct effects on glutamatergic neuronal circuits in

these three laminae, which are innervated by different subpopulations of primary afferent terminals.

It is most remarkable that at least 40% GAD67-eGFP neurons apparently lack labeling for any $GABA_AR$ subunit on their soma in lamina IIo (Figure 3), despite the abundant α2 and α3 subunit-IR in this sublamina, suggesting that these GABAergic neurons receive only limited perisomatic GABAergic inhibitory input. As noted above, our study provides no data on cells selectively expressing GAD65 in the spinal cord. Such cells are rare in the forebrain, where GAD67-eGFP labels the vast majority of GABAergic cells (Tamamaki et al., 2003). In case of spinal cord, GAD65 has been shown to be expressed in a specific GABAergic interneuronal population, which is specifically presynaptic to the proprioceptive terminals (Betley et al., 2009; Hughes et al., 2005).

Glycinergic neurons, which are rare in lamina IIo but abundant in lamina III, widely express the α3 subunit, along with either α2 in lamina IIo, or, in equal proportion α1, α2 or α5 subunit in lamina III. These features provide a substrate for possible cross-talk between the two inhibitory systems.

Functional organization of $GABA_A$ receptor subtypes on primary afferent terminals

Primary afferent depolarization is well known to occur in spinal cord, and $GABA_AR$ have been shown long ago to mediate the inhibition of potassium-stimulated CGRP release in spinal cord tissue (Bourgoin et al., 1992). Overall, the presence of presynaptic $GABA_AR$ in major brain regions, including cerebral cortex, hippocampus, and cerebellum, is undisputed (Hutcheon et al., 2000; Trigo et al., 2010; Vautrin et al., 1994). However, immunohistochemical studies have largely failed so far to reveal their distribution and subunit composition. Therefore, the spinal cord appears to be unique for the prominent labeling of multiple $GABA_AR$ subunits in terminals of both myelinated and non-myelinated sensory afferents. The present study confirms our previous observations (Knabl et al., 2008; Witschi et al., 2011) and provides proof-of-principle evidence that these $GABA_AR$ correspond, at least in part, to axo-axonic synapses, with a postsynaptic density containing the scaffolding protein gephyrin. Moreover, our results show an IR pattern of the various α subunit variants compatible with a complementary distribution (one $GABA_AR$ subtype per afferent terminal); C-fiber terminals containing

either α2- or α3-$GABA_AR$, whereas myelinated terminals in layer III containing in roughly equal proportion one of the four diazepam-sensitive $GABA_AR$ subtypes. These results are compatible with the notion that distinct $GABA_AR$ subtypes modulate the function of primary afferent carrying specific pain modalities and/or being differentially involved in various forms of chronic pain (Zeilhofer et al., 2012a; Zeilhofer et al., 2009). Moreover, while diazepam can be expected to potentiate the action of $GABA_AR$ in primary afferents, subtype-selective ligands would potentially exert more selective effects.

There is increasing evidence for the existence of segregated circuits mediating specific pain-related modalities in the dorsal horn (Neumann et al., 2008; Todd, 2010; Zylka et al., 2005). For instance, a differential distribution of μ- and δ-opioid receptors in subsets of peptidergic and non-peptidergic nociceptors, respectively, regulating distinct pain modalities (heat and mechanical pain) has been reported (Scherrer et al., 2009). TRPV1 receptors, critical for the development of thermal hyperalgesia, also have been found to be restricted to a subset of peptidergic nociceptors in lamina IIo (Cavanaugh et al., 2011) whereas TRPV2, which is expressed non-peptidergic and myelinated nociceptors terminating in lamina IIi and in deeper laminae was conclusively shown to be dispensable for progression of thermal hyperalgesia (Park et al., 2011). In line with these observations, we have shown that mice lacking α2-$GABA_AR$ specifically in primary nociceptors (sns-$\alpha2^{-/-}$ mice) exhibit reduced potentiation of dorsal root potentials and impaired thermal and mechanical anti-hyperalgesia by diazepam in a model of inflammatory pain (Witschi et al., 2011).

The role of α3-$GABA_AR$ in lamina II neurons and primary afferents for the control of hyperalgesia is less well established. They contribute in part to thermal and mechanical hyperalgesia in models of inflammatory and neuropathic pain, possibly along with a contribution by α5-$GABA_AR$ (Knabl et al., 2008; Munro et al., 2011). Finally, based on their distribution on myelinated fibers and lamina III neurons, α5-$GABA_AR$ could have a major role in regulation of allodynia.

The relevance of our finding possibility extends beyond pain control, notably if primary afferent depolarization were involved in other pathological sensory modalities, such as

itch. Both C-fibers and Aδ-fibers have been shown to convey this sensation elicited by histamine (Andrew and Craig, 2001; Potenzieri and Undem, 2012; Schmelz et al., 1997). In addition, decreased inhibitory synaptic input to nociceptive terminals in lamina I-II elicits itch (Ross et al., 2010), in line with our findings.

Conclusions

Collectively, the present results show that $GABA_AR$ distribution in the primary afferent terminals and intrinsic dorsal horn neurons of the dorsal horn allows for multiple, circuit-specific modes of regulation of neuronal networks. This conclusion confirms previous evidence for a differential contribution of α2- and α2/α3/α5-$GABA_AR$ for thermal and mechanical anti-hyperalgesia, respectively (Knabl et al., 2008). The likely existence of parallel and serial connections involving GABAergic neurons allows for both inhibition and disinhibition in a pain modality-specific manner. This organization pattern of $GABA_ARs$ in the adult spinal cord might be altered morphologically and functionally in chronic pain conditions. Identifying these alterations in mouse models of chronic inflammatory and neuropathic pain, will facilitate the design of novel therapeutic avenues based on $GABA_AR$ subtype-selective ligands.

Acknowledgements

We are grateful to Dr. Yuchio Yanagawa (Gunma University Graduate School of Medicine, Maebashi, Japan) for providing GAD67-eGFP mice and Dr. Dietmar Benke, Louis Scheurer, and Isabelle Camenisch for help with biochemical experiments, real time PCR and mouse genotyping. The authors have no competing interests to declare.

References

Andrew D, Craig AD. 2001. Spinothalamic lamina I neurones selectively responsive to cutaneous warming in cats. J Physiol 537:489-495.

Bellocchio EE, Reimer RJ, Fremeau RT, Jr., Edwards RH. 2000. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science 289:957-960.

Bencsits E, Ebert V, Tretter V, Sieghart W. 1999. A significant part of native g-aminobutyric acid$_A$ receptors containing a4 subunits do not contain g or d subunits. J Biol Chem 274:19613-19616.

Betley JN, Wright CV, Kawaguchi Y, Erdelyi F, Szabo G, Jessell TM, Kaltschmidt JA. 2009. Stringent specificity in the construction of a GABAergic presynaptic inhibitory circuit. Cell 139:161-174.

Bogen O, Dreger M, Gillen C, Schroder W, Hucho F. 2005. Identification of versican as an isolectin B4-binding glycoprotein from mammalian spinal cord tissue. FEBS J 272:1090-1102.

Bohlhalter S, Weinmann O, Mohler H, Fritschy JM. 1996. Laminar compartmentalization of GABA$_A$-receptor subtypes in the spinal cord. J Neurosci 16:283-297.

Bourgoin S, Pohl M, Benoliel JJ, Mauborgne A, Collin E, Hamon M, Cesselin F. 1992. g-Aminobutyric acid, through GABA$_A$ receptors, inhibits the potassium-stimulated release of calcitonin gene-related peptide- but not that of substance P-like material from rat spinal cord slices. Brain Res 583:344-348.

Brünig I, Suter A, Knuesel I, Luscher B, Fritschy JM. 2002. GABAergic presynaptic terminals are required for postsynaptic clustering of dystrophin, but not of GABA$_A$ receptors and gephyrin. J Neurosci 22:4805-4813.

Cavanaugh DJ, Chesler AT, Bráz JM, Shah NM, Julius D, Basbaum AI. 2011. Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. J Neurosci 31:10119-10127.

Chang HM, Wei IH, Tseng CY, Lue JH, Wen CY, Shieh JY. 2004. Differential expression of calcitonin gene-related peptide (CGRP) and choline acetyltransferase (ChAT) in the axotomized motoneurons of normoxic and hypoxic rats. J Chem Neuroanat 28:239-251.

Clavier N, Lombard MC, Besson JM. 1992. Benzodiazepines and pain: effects of midazolam on the activities of nociceptive non-specific dorsal horn neurons in the rat spinal cord. Pain 48:61-71.

Dirmeier M, Capellino S, Schubert T, Angele P, Anders S, Straub RH. 2008. Lower density of synovial nerve fibres positive for calcitonin gene-related peptide relative to substance P in rheumatoid arthritis but not in osteoarthritis. Rheumatol 47:36-40.

Eaton MJ, Martinez MA, Karmally S. 1999. A single intrathecal injection of GABA permanently reverses neuropathic pain after nerve injury. Brain Res 835:334-339.

Encinas JM, Vaahtokari A, Enikolopov G. 2006. Fluoxetine targets early progenitor cells in the adult brain. Proc Natl Acad Sci U S A 103:8233-8238.

Fremeau RT, Voglmaier S, Seal RP, Edwards RH. 2004. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. Trends Neurosci 27:98-103.

Fritschy JM, Benke D, Johnson DK, Mohler H, Rudolph U. 1997. $GABA_A$-receptor a-subunit is an essential prerequisite for receptor formation in vivo. Neurosci 81:1043-1053.

Fritschy JM, Brunig I. 2003. Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98:299-323.

Fritschy JM, Panzanelli P, Kralic JE, Vogt KE, Sassoè-Pognetto M. 2006. Differential dependence of axo-dendritic and axo-somatic GABAergic synapses on $GABA_A$ receptors containing the a1 subunit in Purkinje cells. J Neurosci 26:3245-3255.

Fritschy JM, Weinmann O, Wenzel A, Benke D. 1998. Synapse-specific localization of NMDA and $GABA_A$ receptor subunits revealed by antigen-retrieval immunohistochemistry. J Comp Neurol 390:194-210.

Fujiyama F, Furuta T, Kaneko T. 2001. Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex. J Comp Neurol 435:379-387.

Gibbins IL, Wattchow D, Coventry B. 1987. Two immunohistochemically identified populations of calcitonin gene-related peptide (CGRP)-immunoreactive axons in human skin. Brain Res 414:143-148.

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N. 2003. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature 425:917-925.

Hughes DI, Mackie M, Nagy GG, Riddell JS, Maxwell DJ, Szabó G, Erdélyi F, Veress G, Szucs P, Antal M, Todd AJ. 2005. P boutons in lamina IX of the rodent spinal cord

express high levels of glutamic acid decarboxylase-65 and originate from cells in deep medial dorsal horn. Proc Natl Acad Sci U S A 102:9038-9043.

Hunt SP, Mantyh PW. 2001. The molecular dynamics of pain control. Nature Rev Neurosci 2:83-91.

Hutcheon B, Brown LA, Poulter MO. 2000. Digital analysis of light microscope immunofluorescence: high-resolution co-localization of synaptic proteins in cultured neurons. J Neurosci Met 96:1-9.

Jasmin L, Rabkin SD, Granato A, Boudah A, Ohara PT. 2003. Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. Nature 424:316-320.

Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Mohler H, Zeilhofer HU. 2008. Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. Nature 451:330-334.

Kralic JE, Sidler C, Parpan F, Homanics GE, Morrow AL, Fritschy JM. 2006. Compensatory alteration of inhibitory synaptic circuits in cerebellum and thalamus of g-aminobutyric acid type A receptor a1 subunit knockout mice. J Comp Neurol 495:408-421.

Labrakakis C, Lorenzo L, Bories C, Ribeiro-da-Silva A, De Koninck Y. 2009. Inhibitory coupling between inhibitory interneurons in the spinal cord dorsal horn. Mol Pain 5:24:1-8.

Ma W, Saunders PA, Somogyi R, Poulter MO, Barker JL. 1993. Ontogeny of $GABA_A$ receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. J Comp Neurol 338:337-359.

Melzack, R, Wall PD. 1965. Pain mechanisms: A new theory. Science 150:971-978.

Möhler H. 2006. $GABA_A$ receptor diversity and pharmacology. Cell Tissue Res 326:505-516.

Munro G, Erichsen HK, Rae MG, Mirza NR. 2011. A question of balance--positive versus negative allosteric modulation of $GABA_A$ receptor subtypes as a driver of analgesic efficacy in rat models of inflammatory and neuropathic pain. Neuropharmacol 61.

Nahmani M, Erisir A. 2005. VGluT2 immunochemistry identifes thalamocortical terminals in layer 4 of adult and developing visual cortex. J Comp Neurol 484:458-473.

Neumann S, Braz JM, Skinner K, Llewellyn-Smith IJ, Basbaum AI. 2008. Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. J Neurosci 28:7936-7944.

Olsen RW, Sieghart W. 2008. International Union of Pharmacology. LXX. Subtypes of g-aminobutyric acid$_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 60:243.

Panzanelli P, Bardy C, Nissant A, Pallotto M, Sassoè-Pognetto M, Lledo PM, Fritschy JM. 2009. Early synapse formation in developing interneurons of the adult olfactory bulb. J Neurosci 29:15039-15052.

Panzanelli P, Gunn BG, Schlatter MC, Benke D, Tyagarajan SK, Scheiffele P, Belelli D, Lambert JJ, Rudolph U, Fritschy JM. 2011. Distinct mechanisms regulate GABA$_A$ receptor and gephyrin clustering at perisomatic and axo-axonic synapses on CA1 pyramidal cells. J Physiol 589:4959-4980.

Park U, Vastani N, Guan Y, Raja SN, Koltzenburg M, Caterina MJ. 2011. TRP vanilloid 2 knock-out mice are susceptible to perinatal lethality but display normal thermal and mechanical nociception. J Neurosci 31:11425-11436.

Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, Houser CR. 2002. GABA$_A$ receptor changes in d subunit-deficient mice: altered expression of a4 and g2 subunits in the forebrain. J Comp Neurol 446:179-197.

Persohn E, Malherbe P, Richards JG. 1991. In situ hybridization histochemistry reveals a diversity of GABA$_A$ receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. Neurosci 42:497-507.

Persohn E, Malherbe P, Richards JG. 1992. Comparative molecular neuroanatomy of cloned GABA$_A$ receptor subunits in the rat CNS. J Comp Neurol 326:193-216.

Pfeiffer F, Simler R, Grenningloh G, Betz H. 1984. Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of rat spinal cord. Proc Natl Acad Sci U S A 81:7224-7227.

Potenzieri C, Undem BJ. 2012. Basic mechanisms of itch. Clin Exp Allergy 42:8-19.

Rexed B. 1952. The cytoarchitectonic organization of the spinal cord in the cat. J Comp Neurol 96:415-495.

Ribeiro-da-Silva A, Coimbra A. 1982. Two types of synaptic glomeruli and their distribution in laminae I-III of the rat spinal cord. J Comp Neurol 209:176-186.

Ribeiro-da-Silva A, Pignatelli D, Coimbra A. 1985. Synaptic architecture of glomeruli in superficial dorsal horn of rat spinal cord, as shown in serial reconstructions. J Neurocytol 14:203-220.

Roberts LA, Beyer C, Komisaruk BR. 1986. Nociceptive responses to altered GABAergic activity at the spinal cord. Life Sci 39:1667-1674.

Ross SE, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok SI, Shah A, Savner EM, Tolias C, Corfas R, Chen S, Inquimbert P, Xu Y, McInnes RR, Rice FL, Corfas G, Ma Q, Woolf CJ, Greenberg ME. 2010. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. Neuron 65:886-898.

Rudolph U, Crestani F, Mohler H. 2001. $GABA_A$ receptor subtypes: dissecting their pharmacological functions. Trends Pharmacol Sci 22:188-194.

Rudolph U, Mohler H. 2006. GABA-based therapeutic approaches: $GABA_A$ receptor subtype functions. Curr Opin Pharmacol 6:18-23.

Sassoè-Pognetto M, Panzanelli P, Sieghart W, Fritschy JM. 2000. Co-localization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. J Comp Neurol 420:481-498.

Sastry BR. 1980. Potentiation of presynaptic inhibition of nociceptive pathways as a mechanism for analgesia. Can J Physiol Pharmacol 58:97-101.

Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, Kieffer BL, Basbaum AI. 2009. Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. Cell 137:1148-1159.

Schmalfeldt M, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR. 1998. Versican V2 is a major extracellular matrix component of the mature bovine brain. J Biol Chem 273:15758-15764.

Schmelz M, Luz O, Averbeck B, Bickel A. 1997. Plasma extravasation and neuropeptide release in human skin as measured by intradermal microdialysis. Neurosci Lett 230:117-120.

Schneider Gasser EM, Duveau V, Prenosil GA, Fritschy JM. 2007. Reorganization of GABAergic circuits maintains $GABA_A$ receptor-mediated transmission onto CA1 interneurons in a1-subunit-null mice. Eur J Neurosci 25:3287-3304.

Schneider Gasser EM, Straub CJ, Panzanelli P, Weinmann O, Sassoe-Pognetto M, Fritschy JM. 2006. Immunofluorescence in brain sections: simultaneous detection

of presynaptic and postsynaptic proteins in identified neurons. Nat Protoc 1:1887-1897.

Sieghart W. 2006. Structure, pharmacology, and function of $GABA_A$ receptor subtypes. Adv Pharmacol 54:231-263.

Studer R, von Boehmer L, Haenggi T, Schweizer C, Benke D, Rudolph U, Fritschy JM. 2006. Alteration of GABAergic synapses and gephyrin clusters in the thalamic reticular nucleus of $GABA_A$ receptor a3 subunit-null mice. Eur J Neurosci 24:1307-1315.

Sumida T, Tagami M, Ide Y, Nagase M, Sekiyama H, Hanaoka K. 1995. Intravenous midazolam suppresses noxiously evoked activity of spinal wide dynamic range neurons in cats. Anesth Analg 80:58.

Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R, Myers J, Atack JR, Dawson GR, McKernan RM, Whiting PJ, Rosahl TW. 2001. Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. J Neurosci 21:3409-3418.

Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. 2003. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J Comp Neurol 467:60-79.

Todd AJ. 1996. GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. Eur J Neurosci 8:2492-2498.

Todd AJ. 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11:823-836.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ. 2003. The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur J Neurosci 17:13-27.

Trigo FF, Bouhours B, Rostaing P, Papageorgiou G, Corrie JE, Triller A, Ogden D, Marty A. 2010. Presynaptic miniature GABAergic currents in developing interneurons. Neuron 66:235-247.

Vautrin J, Schaffner AE, Barker JL. 1994. Fast presynaptic $GABA_A$ receptor-mediated Cl^- conductance in cultured rat hippocampal neurones. J Physiol 479 (Pt 1):53-63.

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE. 2001. $GABA_A$ receptor a1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. J Neurosci 21:3009-3016.

Wang Y, Kakizaki T, Sakagami H, Saito K, Ebihara S, Kato M, Hirabayashi M, Saito Y, Furuya N, Yanagawa Y. 2009. Fluorescent labeling of both GABAergic and glycinergic neurons in vesicular GABA transporter (VGAT)-venus transgenic mouse. Neurosci 164:1031-1043.

Wieland HA, Lüddens H, Seeburg PH. 1992. A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426.

Wisden W, Gundlach AL, Barnard EA, Seeburg PH, Hunt SP. 1991. Distribution of $GABA_A$ receptor subunit mRNAs in rat lumbar spinal cord. Mol Brain Res 10:179-183.

Witschi R, Punnakkal P, Paul J, Walczak JS, Cervero F, Fritschy JM, Kuner R, Keist R, Rudolph U, Zeilhofer HU. 2011. Presynaptic a2-$GABA_A$ receptors in primary afferent depolarization and spinal pain control. J Neurosci 31:8134.

Yaksh TL. 1989. Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. Pain 37:111-123.

Zeilhofer HU, Benke D, Yévenes GE. 2012a. Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. Annu Rev Pharmacol Toxicol 52:111-133.

Zeilhofer HU, Möhler H, Di Lio A. 2009. GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. Trends Pharmacol Sci 30:397-402.

Zeilhofer HU, Studler B, Arabadzisz D, Schweizer C, Ahmadi S, Layh B, Bosl MR, Fritschy JM. 2005. Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. J Comp Neurol 482:123-141.

Zeilhofer HU, Wildner H, Yévenes GE. 2012b. Fast synaptic inhibition in spinal sensory processing and pain control. Physiol Rev 92:193-235.

Zimmermann DR, Dours-Zimmermann MT, Schubert M, Bruckner-Tuderman L. 1994. Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. J Cell Biol 124:817-825.

Zylka MJ, Rice FL, Anderson DJ. 2005. Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45:17-25.

4 Discussion and Future Perspectives

The studies presented in this thesis address the contribution of spinal $GABA_AR$ subtypes to BDZ-induced analgesia. Results from morphological, electrophysiological and behavioral experiments provide compelling evidence for the existence of a genuine analgesic, or better antihyperalgesic, action of spinal α2 $GABA_AR$. Morphological experiments showed that spinal nociceptor terminals express α2 $GABA_AR$ together with α3 $GABA_AR$. Specific ablation of the $GABA_AR$ α2 subunits from these nociceptor terminals partially prevented the antihyperalgesic action of intrathecally applied diazepam (chapter 3.1), while complete ablation of $GABA_AR$ α2 subunits from the spinal cord and periphery completely blocked the α2 $GABA_AR$-dependent component of antihyperalgesia by the novel non-sedative BDZ site ligand HZ166 (chapter 3.2). These results are in perfect agreement with previous studies from our group (Knabl et al., 2008), which attributed to α2 $GABA_AR$ a critical role in the spinal antihyperalgesic effects of intrathecally injected diazepam in inflammatory and neuropathic pain states – at least in rodents.

While the dominant contribution of α2 $GABA_AR$ is widely accepted (Knabl et al., 2008; Knabl et al., 2009; Munro et al., 2009), it is less clear whether additional activity at α3 or α5 $GABA_AR$ is also needed (Munro et al., 2011). In two previous publications (Knabl et al., 2008, Knabl et al., 2009), our group has reported that α3 and α5 $GABA_AR$ also contribute to antihyperalgesia but less than α2 $GABA_AR$ and that their contribution varies depending on the sensory quality (heat, cold or mechanical) and on the type of pain model used (zymosan A, chronic constriction injury and formalin). In these studies, the contribution of α2 $GABA_AR$ was strongest for relief from heat hyperalgesia both after inflammation and neuropathy consistent with the prominent expression of $GABA_AR$ α2 subunits on nociceptor terminals in lamina IIo, as demonstrated in chapter 3.3 of this thesis. By contrast, myelinated sensory terminals in the deeper laminae, whose activation is responsible for touch-evoked pain (allodynia), expressed mainly α5 and α3 subunits. In line with this differential expression, α3 and α5 $GABA_AR$ made stronger contributions to antihyperalgesia against mechanical allodynia than heat hyperalgesia (Knabl et al., 2008). So far, our conclusions on the contribution of the different $GABA_AR$ subtypes

were mainly based on the loss of antihyperalgesic activity in $GABA_AR$ point-mutated or knock-out mice. These experiments provided important information on the $GABA_AR$ necessary for antihyperalgesia. It is however difficult to conclude from these experiments whether additional efficacy at α3 and α5 $GABA_AR$ would provide better antihyperalgesia than activity at α2 $GABA_AR$ alone. Work with the recently developed subtype-selective agonist NS 11394 (Mirza et al., 2008) points at a significant contribution of α5 $GABA_AR$ to antihyperalgesia (Munro et al., 2008). At present there are not enough subtype-selective compounds available that would allow addressing all possible subunit combinations. Alternatively, the generation of mice carrying point mutations in more than one α subunit would allow clarifying this issue in reasonable time span.

The results of such studies will have important implications for the development of novel therapeutic approaches to chronic pain. Since several years, drug companies are working on the development of novel subtype-specific $GABA_AR$ modulators with improved tolerability or novel therapeutic indications (Griebel et al., 2001; Knabl et al., 2009; Munro et al., 2008; Nickolls et al., 2011). Several such compounds have been developed in the last decade demonstrating that subtype-selective targeting of $GABA_AR$ is possible in principle. Most of these drug discovery and drug development programs aimed at compounds with improved selectivity at α2 and α3 $GABA_AR$ over α1 $GABA_AR$. In most cases, the development of non-sedative anxiolytic drugs was the primary intention. However, several of these subtype-selective BDZ site ligands have meanwhile also been tested for potential antihyperalgesic activity in a wide variety of rodent pain models (Di Lio et al., 2011; Knabl et al., 2008; Munro et al., 2008; Nickolls et al., 2011). Most of these compounds exhibited significant antihyperalgesic effects in rat and mouse neuropathic pain models (tab. 1). In some studies, the efficacy reached was similar to that of gabapentin or pregabalin, drugs frequently used for the treatment of painful neuropathies in human patients (Di Lio et al., 2011; Nickolls et al., 2011). For several of these compounds, efficacy was also seen in inflammatory models, but efficacy was generally less than in neuropathic models. Comparison of the antihyperalgesic and anxiolytic efficacies of several subtype-selective compounds suggests that antihyperalgesia requires, in general, higher intrinsic activity than anxiolysis. This was also obvious from a previous study of our group (Knabl et al., 2009), which showed antihyperalgesic and sedative actions of systemic diazepam occur with similar dose-dependencies, while anxiolysis can be reached already at 10-fold lower doses.

The positive results from our rodent studies and from those of other groups are in apparent contrast to clinical experience with classical BDZ. Although there are several anecdotal reports of beneficial effects in chronic pain patients (Tucker et al., 2004), classical BDZ do in general not exert analgesic actions after systemic treatment. Treatment guidelines include them typically as co-medications for their anxiolytic and muscle relaxant effects (Gold and Dribben, 1964; Højsted et al., 2010; Khanderia and Pandit, 1987; Nordbø et al., 2012).

There are several possible explanations for the discrepancies between the promising results from rodent studies and the lack of evidence from clinical experience in human pain patients. Although species differences in the distribution and the function of $GABA_AR$ subtypes in nociceptive pathways cannot be excluded at present, a recent study by Waldvogel et al. (2010) did not reveal major differences in the brain. An alternative explanation could be the higher susceptibility of the human brain to the sedative effects of BDZ site agonists. It has in fact been demonstrated that several compounds with reduced efficacy at α1 $GABA_AR$ caused sedation in human volunteers, although such sedative effects had not been found in preclinical rodent studies (De Haas et al., 2007). Antihyperalgesic action of classical non-selective BDZ might therefore only occur at concentrations, which induce already strong sedation in humans. Compounds suitable as antihyperalgesic agents for human treatment should therefore have a high intrinsic activity at α2 $GABA_AR$ (and perhaps also at α3 or α5 $GABA_AR$) but very little activity at α1 $GABA_AR$. Such compounds are not yet available for human testing.

Part of the discrepancy of preclinical rodent data and clinical experience in human patients might also come from the different routes of administration. In most of our studies (including the study described in chapter 3.1 of this thesis) diazepam was given locally to the spinal cord, whereas under clinical conditions BDZ are usually given systemically. A few studies performed with intrathecal midazolam did in fact find evidence for the existence of BDZ-mediated analgesia also in human patients (Prochazka et al., 2011; Tucker et al., 2004). It is therefore possible that supraspinal $GABA_AR$ might counteract a spinal antihyperalgesic effect of BDZ. Results from chapter 3.2 of this thesis argue against a significant effect of supraspinal α2 $GABA_AR$ on antihyperalgesia, but similar evidence is not yet available for the other $GABA_AR$ subtypes.

L-838417 which almost completely lacks activity at α1 $GABA_AR$ and which exhibits moderate activity at α2, α3, and α5 $GABA_AR$, is antihyperalgesic in different rodent models of inflammatory and neuropathic pain (Knabl et al., 2008; Nickolls et al., 2011), but has very poor pharmacokinetic properties in man preventing its testing in human volunteers or patients (tab.1). Another highly selective compound is TPA023 (low intrinsic activity at α2 and α3 $GABA_AR$, negligible activity at α1 and α5 $GABA_AR$ shows only moderate antihyperalgesic activity in some but not all rodent pain models most likely due to its low intrinsic activity (Nickolls et al., 2011). HZ166 is another recently developed BDZ site ligand with high activity at α2 and α5 $GABA_AR$ but reduced sedative properties (Rivas et al., 2009; Fischer et al., 2010; Di Lio et al., 2011). In mice it exerts antihyperalgesic activity comparable to that of non-sedative doses of gabapentin. This compound has not yet gone through human tolerability screening and is therefore not available for human studies. Whether its therapeutic window is sufficiently broad to allow antihyperalgesic activity in the absence of sedation is not clear.

The question whether subtype-selective $GABA_AR$ agonists exert antihyperalgesic activity in humans cannot be answered conclusively at this stage but will depend on the future availability of compounds with high intrinsic activity at α2 $GABA_AR$ (and perhaps α3 and α5 $GABA_AR$) and high selectivity over α1 $GABA_AR$ in addition to suitable pharmacokinetic properties and good tolerability in humans. Hopefully such compounds will become available soon from on-going drug development programs at several pharmaceutical and biotechnological companies (Rogawski, 2006).

In summary, using the classical BDZ, diazepam, and the more recently developed BDZ site ligand, HZ166, and a combination of anatomical, electrophysiological and behavioral tools, this dissertation has provided proof-of-principle evidence that spinal α2 $GABA_AR$, which are abundant in the primary afferent terminals and dorsal horn interneurons, are the predominant contributors to the antihyperalgesic actions of $GABA_AR$.

Compound	Relative potentiation (%)				Reference	Effects in pain models
	α1	α2	α3	α4		
NS11394	7.8	26	52	78	Munro et al., 2008	antinociceptive in rat formalin and capsaicin test; antihyperalgesic in CFA inflammation, CCI and SNI.
HZ166*	27	52	36	23	Di Lio et al., 2011	antihyperalgesic in mouse zymosan A and CCI
SL651498	70	>100	83	50	Griebel et al., 2001 Knabl et al., 2009	reduced electrically evoked flexor responses in rats; antinociceptive in mouse formalin test
L-838,417	1	15	12	32	Knabl et al., 2008 Nickolls et al., 2011	antihyperalgesic in rat zymosan A and CCI, antiallodynic in rat SNL but not TNT; antihyperalgesic but no antiallodynic effect in rat CFA
TPA023	0.6	4	9	5	Nickolls et al., 2011	antiallodynic in rat SNL; no antihyperalgesic effect in rat CFA
Zolpidem	153	75	78	13	Munro et al., 2008	effective in rat formalin and capsaicin, but only at sedative doses
Bretazenil	73	21	33	42	Munro et al., 2008	no antihyperalgesia in rat CCI and SNI at non-sedative doses

*Compound 2 in Rivas et al., 2009

Tab 1. Subtype selectivity of BDZ Site Ligands.

5 References

Abe K, Kato G, Katafuchi T, Tamae A, Furue H, Yoshimura M (2009) Responses to 5-HT in morphologically identified neurons in the rat substantia gelatinosa in vitro. Neuroscience 159:316-324.

Abrahamsen B, Zhao J, Asante CO, Cendan CM, Marsh S, Martinez-Barbera JP, Nassar MA, Dickenson AH, Wood JN (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. Science 321:702-705.

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002) PGE2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 5:34-40.

Al Khater KM, Todd AJ (2009) Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. J Comp Neurol 515:629-646.

Antal M, Petko M, Polgar E, Heizmann CW, Storm-Mathisen J (1996) Direct evidence of an extensive GABAergic innervation of the spinal dorsal horn by fibres descending from the rostral ventromedial medulla. Neuroscience 73:509-518.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267-284.

Beiche F, Scheuerer S, Brune K, Geisslinger G, Goppelt-Struebe M (1996) Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation. FEBS letters 390:165-169.

Belelli D, Peden DR, Rosahl TW, Wafford KA, Lambert JJ (2005) Extrasynaptic $GABA_A$ receptors of thalamocortical neurons: a molecular target for hypnotics. J Neurosci 25:11513-11520.

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL (1997) $GABA_A$, NMDA and AMPA receptors: a developmentally regulated ménage trois'. Trends Neurosci 20:523-529.

Benke D, Cicin-Sain A, Mertens S, Mohler H (1991) Immunochemical identification of the alpha 1- and alpha 3-subunits of the $GABA_A$-receptor in rat brain. J Recept Res. 11:407-424.

Bennett GJ, Xie YKA (1988) Peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87–107.

Benson JA, Löw K, Keist R, Mohler H, Rudolph U (1998) Pharmacology of recombinant [gamma]-aminobutyric acidA receptors rendered diazepam-insensitive by point-mutated [alpha]-subunits. FEBS letters 431:400-404.

Beyer C, Roberts LA, and Komisaruk BR (1985) Hyperalgesia induced by altered glycinergic activity at the spinal cord. Life Sci 37: 875-882.

Bianchi MT, Haas KF, Macdonald RL (2002) Alpha1 and alpha6 subunits specify distinct desensitization, deactivation and neurosteroid modulation of $GABA_A$ receptors containing the delta subunit. Neuropharmacology 43:492-502.

Bohlhalter S, Weinmann O, Mohler H, Fritschy JM (1996) Laminar compartmentalization of $GABA_A$-receptor subtypes in the spinal cord: an immunohistochemical study. J Neurosci 16:283-297.

Bowery NG, Hill DR, Hudson AL, Doble A, Middlemiss DN, Shaw J, Turnbull M (1980) (–) Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. Nature 283:92-94.

Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D (2006) Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur J Pain 10:287-333.

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. J Physiol 497:753-759.

Brooks J, Tracey I (2005) From nociception to pain perception: imaging the spinal and supraspinal pathways. Journal of anatomy 207:19-33.

Bushnell MC, Apkarian AV (2006) Representation of pain in the brain. Wall and Melzack's textbook of pain:107-124.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306-313.

Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci 106:9075-9080.

Cheng VY, Bonin RP, Chiu MW, Newell JG, MacDonald JF, Orser BA (2006) Gabapentin increases a tonic inhibitory conductance in hippocampal pyramidal neurons. Anesthesiology 105:325-333.

Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CBA, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN (2005) Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 114:386-396.

Claveau D, Sirinyan M, Guay J, Gordon R, Chan CC, Bureau Y, Riendeau D, Mancini JA (2003) Microsomal prostaglandin E synthase-1 is a major terminal synthase that is selectively up-regulated during cyclooxygenase-2-dependent prostaglandin E2 production in the rat adjuvant-induced arthritis model. J Immunol 170:4738.

Clavier N, Lombard MC, Besson JM (1992) Benzodiazepines and pain: effects of midazolam on the activities of nociceptive non-specific dorsal horn neurons in the rat spinal cord. Pain 48:61-71.

Coderre TJ, Kumar N, Lefebvre CD, Yu JS (2005) Evidence that gabapentin reduces neuropathic pain by inhibiting the spinal release of glutamate. J Neurochem 94:1131-1139.

Coull JAM, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, Koninck PD, Koninck YD (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424:938-942.

Coull JAM, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438:1017-1021.

Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes congenital inability to experience pain. Nature 444:894-898.

Crestani F, Löw K, Keist R, Mandelli MJ, Möhler H, Rudolph U (2001) Molecular targets for the myorelaxant action of diazepam. Mol Pharmacol 59:442-445.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Blüthmann H, Möhler H, Rudolph U (2002) Trace fear conditioning involves hippocampal alpha5 $GABA_A$ receptors. Proc Natl A Sci U S A 99:8980-8985.

De Haas SL, De Visser SJ, Van Der Post JP, De Smet M, Schoemaker RC, Rijnbeek B, Cohen AF, Vega JM, Agrawal NGB, Goel TV (2007) Pharmacodynamic and pharmacokinetic effects of TPA023, a $GABA_A$ 2, 3 subtype-selective agonist, compared to lorazepam and placebo in healthy volunteers. Journal of Psychopharmacology 21:374-383.

Decosterd I, Woolf CJ (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87:149-158.

Dhaka A, Earley TJ, Watson J, Patapoutian A (2008) Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. J Neurosci 28:566-575.

Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ, Edwankar R, Cook JM, Zeilhofer HU (2011) HZ166, a novel $GABA_A$ receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. Neuropharmacology. 60:626-632.

Dixon CI, Morris HV, Breen G, Desrivieres S, Jugurnauth S, Steiner RC, Vallada H, Guindalini C, Laranjeira R, Messas G (2011) Cocaine effects on mouse incentive-learning and human addiction are linked to alpha2 subunit-containing $GABA_A$ receptors. Proc Natl A Sci U S A 107:2289-2294.

Eaton MJ, Martinez MA, Karmally S (1999) A single intrathecal injection of GABA permanently reverses neuropathic pain after nerve injury. Brain Res 835:334-339.

Eaton MJ, Plunkett JA, Karmally S, Martinez MA, Montanez K (1998) Changes in GAD- and GABA-immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplant of immortalized serotonergic precursors. J Chem Neuroanat 16:57-72.

Eccles JC, Eccles RM, Magni F (1961) Central inhibitory action attributable to presynaptic depolarization produced by muscle afferent volleys. J Physiol 159:147-66.

Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the gamma 2 subunit and gephyrin. Nat Neurosci 1:563-571.

Farrant M, Kaila K (2007) The cellular, molecular and ionic basis of $GABA_A$ receptor signalling. Prog Brain Res 160:59-87.

Felix M. Rivas, James P. Stables, Lauren Murphree, Rahul V. Edwankar, Chitra R. Edwankar, Shengming Huang, Hiteshkumar D. Jain, Hao Zhou, Samarpan Majumder, Subramanian Sankar, Bryan L. Roth, Joachim Ramerstorfer, Roman Furtmüller, Werner Sieghart, and James M. Cook (2009) Antiseizure Activity of Novel gama-Aminobutyric Acid (A) Receptor Subtype-Selective Benzodiazepine Analogues in Mice and Rat Models. J. Med. Chem. 52:1795-1798.

Fischer BD, Licata SC, Edwankar RV, Wang ZJ, Huang S, He X, Yu J, Zhou H, Johnson EM Jr, Cook JM, Furtmüller R, Ramerstorfer J, Sieghart W, Roth BL, Majumder S,

Rowlett JK (2010). Anxiolytic-like effects of 8-acetylene imidazobenzodiazepines in a rhesus monkey conflict procedure. Neuropharmacology. 59:612-618.

Fritschy JM, Mohler H (1995) $GABA_A$ receptor heterogeneity in the adult rat brain: Differential regional and cellular distribution of seven major subunits. J Comp Neurol 359:154-194.

Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Mühler H (1992) Five subtypes of type A gamma-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. Proc Natl A Sci U S A 89:6726-6730.

Gassner M, Ruscheweyh R, Sandkuhler J (2009) Direct excitation of spinal GABAergic interneurons by noradrenaline. Pain 145:204-210.

Gauriau C, Bernard JF (2004) Posterior triangular thalamic neurons convey nociceptive messages to the secondary somatosensory and insular cortices in the rat. J Neurosci 24:752-761.

Gee NS, Brown JP, Dissanayake VU, Offord J, Thurlow R, Woodruff GN (1996) The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha2delta subunit of a calcium channel. J Biol Chem 271:5768-5776.

Gold RL, Dribben IS (1964) Diazepam therapy of depressed patients. J Neuropsychiatr. 5:366-369.

Grudt TJ, Perl ER (2002) Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J Physiol 540:189-207.

Günther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M, Lang Y (1995) Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. Proc Natl A Sci U S A 92:7749-7753.

Haas KF, Macdonald RL (1999) $GABA_A$ receptor subunit gamma2 and delta subtypes confer unique kinetic properties on recombinant $GABA_A$ receptor currents in mouse fibroblasts. J Physiol 514:27-45.

Hantman AW, van den Pol AN, Perl ER (2004) Morphological and physiological features of a set of spinal substantia gelatinosa neurons defined by green fluorescent protein expression. J Neurosci 24:836-842.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 32:77-88.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schütz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU, Müller U (2004) GlyR alpha3: An Essential Target for Spinal PGE2-Mediated Inflammatory Pain Sensitization. Science 304:884-887.

Heinemann U, Schaible HG, Schmidt RF (1990) Changes in extracellular potassium concentration in cat spinal cord in response to innocuous and noxious stimulation of legs with healthy and inflamed knee joints. Exp Brain Res 79:283-292.

Herrero JF, Laird J, Lopez-Garcia JA (2000) Wind-up of spinal cord neurones and pain sensation: much ado about something? Prog Neurobiol 61:169-203.

Højsted J, Nielsen PR, Guldstrand SK, Frich L, Sjogren P (2010) Classification and identification of opioid addiction in chronic pain patients. Eur J Pain 14: 1014–1020.

Honore P, Wismer CT, Mikusa J, Zhu CZ, Zhong C, Gauvin DM, Gomtsyan A, El Kouhen R, Lee CH, Marsh K (2005) A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. J Pharmacol Exp Ther 314:410-421.

Hösl K, Reinold H, Harvey RJ, Müller U, Narumiya S, Zeilhofer HU (2006) Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor [alpha] 3 subunit, which mediate central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection. Pain 126:46-53.

Hunkeler W, Mohler H, Pieri L, Polc P, Bonetti EP, Cumin R, Schaffner R, Haefely W (1981) Selective antagonists of benzodiazepines. Nature 290:514-516.

Hu, H. J., Carrasquillo, Y., Karim, F., Jung, W. E., Nerbonne, J. M., Schwarz, T. L., Gereau, R. W. 4th (2006) The kv4.2 potassium channel subunit is required for pain plasticity. Neuron 50:89-100.

Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. Nat Rev Neurosci 2:83-91.

IASP (2010) Declaration of Montreal. Delegates to the International Pain Summit of the International Association for the Study of Pain.

Ibuki T, Hama AT, Wang XT, Pappas GD, Sagen J (1996) Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. Neuroscience 76:845-858.

Ikeda H, Heinke B, Ruscheweyh R, Sandkühler J (2003) Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299:1237-1240.

Ikeda H, Stark J, Fischer H, Wagner M, Drdla R, Jäger T, Sandkühler J (2006) Synaptic amplifier of inflammatory pain in the spinal dorsal horn. Science 312:1659-1662.

Ilyin VI, Pomonis JD, Whiteside GT, Harrison JE, Pearson MS, Mark L, Turchin PI, Gottshall S, Carter RB, Nguyen P, Hogenkamp DJ, Olanrewaju S, Benjamin E, Woodward RM (2006) Pharmacology of 2-[4-(4-chloro-2-fluorophenoxy) phenyl]-pyrimidine-4-carboxamide: a potent, broad-spectrum state-dependent sodium channel blocker for treating pain states. J Pharmacol Exp Ther. 318:1083-1093.

Jacob TC, Bogdanov YD, Magnus C, Saliba RS, Kittler JT, Haydon PG, Moss SJ (2005) Gephyrin regulates the cell surface dynamics of synaptic $GABA_A$ receptors. J Neurosci 25:10469-10478.

Jasmin L, Rabkin SD, Granato A, Boudah A, Ohara PT (2003) Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. Nature 424:316-320.

Kato G, Yasaka T, Katafuchi T, Furue H, Mizuno M, Iwamoto Y, Yoshimura M (2006) Direct GABAergic and glycinergic inhibition of the substantia gelatinosa from the rostral ventromedial medulla revealed by in vivo patch-clamp analysis in rats. J Neurosci 26:1787-1794.

Keller A, Coull JAM, Chéry N, Poisbeau P, De Koninck Y (2001) Region-specific developmental specialization of GABA–glycine cosynapses in laminas I–II of the rat spinal dorsal horn. J Neurosci 21:7871-780. Khanderia U, Pandit SK (1987) Use of midazolam hydrochloride in anesthesia. Clin Pharm. 6:533-547.

Khanderia U, Pandit SK (1987) Use of midazolam hydrochloride in anesthesia. Clin Pharm. 6:533-547.

Kim SH, Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50: 355–363.

Kirsch J, Langosch D, Prior P, Littauer UZ, Schmitt B, Betz H (1991) The 93-kDa glycine receptor-associated protein binds to tubulin. J Biol Chem 266:22242-22245.

Knabl J, Zeilhofer UB, Crestani F, Rudolph U, Zeilhofer HU (2009) Genuine antihyperalgesia by systemic diazepam revealed by experiments in $GABA_A$ receptor point-mutated mice. Pain 141:233-238.

Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Mohler H, Zeilhofer HU (2008)

Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. Nature 451:330-334.

Krogsgaard-Larsen P, Frolund B, Liljefors T, Ebert B (2004) $GABA_A$ agonists and partial agonists: THIP (Gaboxadol) as a non-opioid analgesic and a novel type of hypnotic. Biochem Pharmacol 68:1573-1580.

Kullmann DM, Ruiz A, Rusakov DM, Scott R, Semyanov A, Walker MC (2005) Presynaptic, extrasynaptic and axonal $GABA_A$ receptors in the CNS: where and why? Prog Biophys Mol Biol 87:33-46.

Lagerström MC, Rogoz K, Abrahamsen B, Lind AL, Ölund C, Smith C, Mendez JA, Wallén-Mackenzie Å, Wood JN, Kullander K (2011) A sensory subpopulation depends on vesicular glutamate transporter 2 for mechanical pain, and together with substance P, inflammatory pain. Proc Natl A Sci U S A 108:5789-5794.

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 93:5860-5865.

Lambert JJ, Belelli D (2002) Pharmacological characterization of a novel cell line expressing human alpha(4)beta(3)delta $GABA_A$ receptors: commentary on Brown et al. Br J Pharmacol 136:957-959.

Lamsa K, Taira T (2003) Use-dependent shift from inhibitory to excitatory $GABA_A$ receptor action in SP-O interneurons in the rat hippocampal CA3 area. J Neurophysiol 90:1983-1995.

Lashbrook JM, Ossipova MH, Hunter JC, Raffa RB, Tallarida RJ, Porreca F (1999) Synergistic antiallodynic effects of spinal morphine with ketorolac and selective COX_1- and COX_2-inhibitors in nerve-injured rats. Pain 82:65–72.

Li Z, Taylor CP, Weber M, Piechan J, Prior F, Bian F, Cui M, Hoffman D, Donevan S (2011) Pregabalin is a potent and selective ligand for α(2)δ-1 and α(2)δ-2 calcium channel subunits. Eur J Pharmacol. 667:80-90.

Linley JE, Rose K, Ooi L, Gamper N (2010) Understanding inflammatory pain: ion channels contributing to acute and chronic nociception. Pflügers Archiv Eur J Physiol 459:657-669.

Liu B, Liu Z, Chen T, Li H, Qiang B, Yuan J, Peng X, Qiu M (2007) Selective expression of Bhlhb5 in subsets of early born interneurons and late born association neurons in the spinal cord. Dev Dyn 236:829-835.

Loeser JD, Treede RD (2008) The Kyoto protocol of IASP basic pain terminology. Pain 137:473-477.

Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA, Fritschy JM, Rülicke T, Bluethmann H, Möhler H (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Lu J, Bjorkum AA, Xu M, Gaus SE, Shiromani PJ, Saper CB (2002) Selective activation of the extended ventrolateral preoptic nucleus during rapid eye movement sleep. J Neurosci 22:4568-4576.

Lu Y, Perl ER (2003) A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. J Neurosci 23:8752-8758.

Ma W, Saunders PA, Somogyi R, Poulter MO, Barker JL (1993) Ontogeny of $GABA_A$ receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. J Comp Neurol 338:337-359.

Malmberg AB, Yaksh TL (1992) Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J. Pharmacol. Exp. Ther. 263:136–146.

Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA (1997) Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. Science 278:275-279.

Marais E, Klugbauer N, Hofmann F (2001) Calcium channel alpha(2)delta subunits-structure and Gabapentin binding. Mol Pharmacol 59:1243-1248.

Maxwell DJ, Belle MD, Cheunsuang O, Stewart A, Morris R (2007) Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. J Physiol 584:521-533.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor alpha1 subtype. Nat Neurosci 3:2000-2006.

Meller ST, Gebhart GF (1997) Intraplantar zymosan as a reliable, quantifiable model of thermal and mechanical hyperalgesia in the rat. Eur J Pain 1:43-52.

Melzack R, Wall PD (1965) Pain mechanisms: a new theory. Science 150:971-979.

Mendell LM (1984) Modifiability of spinal synapses. Physiol Rev 64:260-324.

Merskey H, Bogduk N (1994) Classification of Chronic Pain. International Association for the Study of Pain 2 ed. Seattle:3-4.

Mirza NR, Larsen JS, Mathiasen C, Jacobsen TA, Munro G, Erichsen HK, Nielsen AN, Troelsen KB, Nielsen EØ, Ahring PK (2008) NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile], a unique subtype-selective $GABA_A$ receptor positive allosteric modulator: in vitro actions, pharmacokinetic properties and in vivo anxiolytic efficacy. J Pharmacol Exp Ther. 327:954-968.

Möhler H (2006) $GABA_A$ receptor diversity and pharmacology. Cell Tissue Res 326:505-516.

Möhler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. J Pharmacol Exp Ther 300:2-8.

Mokha SS, McMillan JA, Iggo A (1983) Dorsal root potentials in the cat: effects of bicuculline. Brain Res 259:313-318.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci 22:6724-6731.

Moore RA, Wiffen PJ, Derry S, McQuay HJ **(**2011) Gabapentin for chronic neuropathic pain and fibromyalgia in adults. Cochrane Database Syst Rev. 16:CD007938.

Munn EM, Harte SE, Lagman A, Borszcz GS (2009) Contribution of the periaqueductal gray to the suppression of pain affect produced by administration of morphine into the intralaminar thalamus of rat. J Pain 10:426-435.

Munro G, Lopez-Garcia JA, Rivera-Arconada I, Erichsen HK, Nielsen EO, Larsen JS, Ahring PK, and Mirza NR (2008) Comparison of the novel subtype-selective $GABA_A$ receptor positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. J Pharmacol Exp Ther 327: 969-981.

Munro G , Erichsen HK, Rae MG, Mirza NR (2011) A question of balance e Positive versus negative allosteric modulation of $GABA_A$ receptor subtypes as a driver of analgesic efficacy in rat models of inflammatory and neuropathic pain. Neuropharm 61: 121-132.

Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh-ishi S (2000) Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 275:327-383.

Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA (1999) Transmission of chronic nociception by spinal neurons expressing the substance P receptor. Science 286:1558-1561.

Nickolls S, Mace H, Fish R, Edye M, Gurrell R, Ivarsson M, Pitcher T, Tanimoto-Mori S, Richardson D, Sweatman C, Nicholson J, Ward C, Jinks J, Bell C, Young K, Rees H, Moss A, Kinloch R, McMurray G (2011) A Comparison of the α2/3/5 Selective Positive Allosteric Modulators L-838,417 and TPA023 in Preclinical Models of Inflammatory and Neuropathic Pain. Adv Pharmacol Sci. Epub Nov 28.

Nishi S, Minota S, Karczmar AG (1974) Primary afferent neurones: the ionic mechanism of GABA-mediated depolarization. Neuropharm 13:215-219.

Nordbø A, Skurtveit S, Borchgrevink PC, Kaasa S, Fredheim OM (2012) Low-dose transdermal buprenorphine - long-term use and co-medication with other potentially addictive drugs. Acta Anaesthesiol Scand. 56:88-94.

Orban PC, Chui D, Marth JD (1992) Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci U S A 89:6861-6865.

Pan HL, Wu ZZ, Zhou HY, Chen SR, Zhang HM, Li DP (2008) Modulation of pain transmission by G-protein-coupled receptors. Pharmacol Ther 117:141-161.

Persohn E, Malherbe P, Richards JG (1991) In situ hybridization histochemistry reveals a diversity of $GABA_A$ receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. Neuroscience 42:497-507.

Pfeiffer F, Graham D, Betz H (1982) Purification by affinity chromatography of the glycine receptor of rat spinal cord. J Biol Chem 257:9389-9393.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815-850.

Polgar E, Gray S, Riddell JS, Todd AJ (2004) Lack of evidence for significant neuronal loss in laminae I-III of the spinal dorsal horn of the rat in the chronic constriction injury model. Pain 111:144-150.

Polgar E, Hughes DI, Riddell JS, Maxwell DJ, Puskar Z, Todd AJ (2003) Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. Pain 104:229-239.

Polgár E, Al-Khater KM, Shehab S, Watanabe M, Todd AJ (2008) Large projection neurons in lamina I of the rat spinal cord that lack the neurokinin 1 receptor are

densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors. J Neurosci 28:13150-13160.

Price DD (2000) Psychological and neural mechanisms of the affective dimension of pain. Science 288:1769-1772.

Price DD, Hu JW, Dubner R, Gracely RH (1977) Peripheral suppression of first pain and central summation of second pain evoked by noxious heat pulses. Pain 3:57-68.

Prochazka J, Hejcl A, Prochazkova L (2011) Intrathecal midazolam as supplementary analgesia for chronic lumbar pain-15 years' experience. Pain Med. 12:1309-1315.

Prut L, Prenosil G, Willadt S, Vogt K, Fritschy JM, Crestani F (2010) A reduction in hippocampal $GABA_A$ receptor α5 subunits disrupts the memory for location of objects in mice. Genes Brain Behav 9:478-488.

Rees H, Sluka KA, Westlund KN, Willis WD (1995) The role of glutamate and GABA receptors in the generation of dorsal root reflexes by acute arthritis in the anaesthetized rat. J Physiol 484:437-445.

Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Müller U (2005) Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. J Clin Invest 115:673-679.

Reitsma ML, Tranmer JE, Buchanan DM, Vandenkerkhof EG (2011) The prevalence of chronic pain and pain-related interference in the Canadian population from 1994 to 2008. Chron Dis Inj Can 31:157-164.

Rexed B (1952) The cytoarchitectonic organization of the spinal cord in the cat. J Comp Neurol 96:415-495.

Ribeiro-da-Silva A, Coimbra A (1982) Two types of synaptic glomeruli and their distribution in laminae I-III of the rat spinal cord. J Comp Neurol. 209:176-186.

Ribeiro-da-Silva A, Pignatelli D, Coimbra A (1985) Synaptic architecture of glomeruli in superficial dorsal horn of rat spinal cord, as shown in serial reconstructions. J Neurocytol. 14:203-220.

Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S (2009) Antiseizure Activity of Novel -Aminobutyric Acid (A) Receptor Subtype-Selective Benzodiazepine Analogues in Mice and Rat Models. J Med Chem 52:1795-1798.

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K (1999) The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-255.

Roberts LA, Beyer C, and Komisaruk BR (1986) Nociceptive responses to altered GABAergic activity at the spinal cord. Life Sci 39: 1667-1674.

Rogawski MA (2006) Diverse mechanisms of antiepileptic drugs in the development pipeline. Epilepsy Res 69:273-294.

Rudolph U, Crestani F, Mohler H (2001) $GABA_A$ receptor subtypes: dissecting their pharmacological functions. Trends Pharmacol Sci 22:188-194.

Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Möhler H (1999) Benzodiazepine actions mediated by specific -aminobutyric acidA receptor subtypes. Nature 401:796-800.

Ruiz A, Campanac E, Scott RS, Rusakov DA, Kullmann DM (2010) Presynaptic $GABA_A$ receptors enhance transmission and LTP induction at hippocampal mossy fiber synapses. Nat Neurosci 13:431-438.

Salter MW, Henry JL (1991) Responses of functionally identified neurones in the dorsal horn of the cat spinal cord to substance P, neurokinin A and physalaemin. Neuroscience 43:601-610.

Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ (2001) Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410:471-475.

Sandkühler J (2009) Models and mechanisms of hyperalgesia and allodynia. Physiol rev 89:707-758.

Sassoè Pognetto M, Panzanelli P, Sieghart W, Fritschy JM (2000) Colocalization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. J Comp Neurol 420:481-498.

Sassoè Pognetto M, Kirsch J, Grünert U, Greferath U, Fritschy JM, Möhler H, Betz H, Wässle H (1995) Colocalization of gephyrin and $GABA_A$ receptor subunits in the rat retina. J Comp Neurol 357:1-14.

Sauer B, Henderson N (1988a) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A 85:5166-5170.

Sauer B, Henderson N (1988b) The cyclization of linear DNA in Escherichia coli by site specific recombination. Gene 70:331-341.

Sauer B, Whealy M, Robbins A, Enquist L (1987) Site-specific insertion of DNA into a pseudorabies virus vector. Proc Natl Acad Sci U S A 84:9108-9112.

Savic MM, Obradovic DI, Ugresic ND, Cook JM, Yin W, Van Linn M, Bokonjic DR (2006) Benzodiazepine site inverse agonists and locomotor activity in rats: bimodal and biphasic influence. Pharmacol Biochem Behav 84:35-42.

Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, Kieffer BL, Basbaum AI (2009) Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. Cell 137:1148-1159.

Schneider Gasser EM, Straub CJ, Panzanelli P, Weinmann O, Sassoe-Pognetto M, Fritschy JM (2006) Immunofluorescence in brain sections: simultaneous detection of presynaptic and postsynaptic proteins in identified neurons. Nat Protoc 1:1887-1897.

Scholz J, Woolf CJ (2007) The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 10:1361-1368.

Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH (2009) Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462:651-655.

Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629-632.

Spike RC, Puskar Z, Andrew D, Todd AJ (2003) A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. Eur J Neurosci 18:2433-2448.

Staud R, Cannon RC, Mauderli AP, Robinson ME, Price DD, Vierck CJ (2003) Temporal summation of pain from mechanical stimulation of muscle tissue in normal controls and subjects with fibromyalgia syndrome. Pain 102:87-95.

Sugimoto T, Bennett GJ, Kajander KC (1990) Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: effects of a chronic constriction injury, transection, and strychnine. Pain 42:205-213.

Sumida T, Tagami M, Ide Y, Nagase M, Sekiyama H, Hanaoka K (1995) Intravenous midazolam suppresses noxiously evoked activity of spinal wide dynamic range neurons in cats. Anesth Analg 80:58-63.

Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R, Myers J, Atack JR, Dawson GR, McKernan

RM, Whiting PJ, Rosahl TW (2001) Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. J Neurosci 21:3409-3418.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS (2002) Hormonally regulated alpha(4)beta(2)delta $GABA_A$ receptors are a target for alcohol. Nat Neurosci 5:721-722.

Sung KW, Kirby M, McDonald MP, Lovinger DM, Delpire E (2000) Abnormal $GABA_A$ receptor-mediated currents in dorsal root ganglion neurons isolated from Na–K–2Cl cotransporter null mice. J Neurosci 20:7531-7538.

Taketo M, Yoshioka T (2000) Developmental change of $GABA_A$ receptor-mediated current in rat hippocampus. Neuroscience 96:507-514.

Tanabe M, Takasu K, Takeuchi Y, Ono H (2008) Pain relief by gabapentin and pregabalin via supraspinal mechanisms after peripheral nerve injury. J Neurosci Res. 86:3258-3264.

Täuber M, Calame-Droz E, Prut L, Rudolph U, Crestani F (2003) α2-γ-Aminobutyric acid $(GABA)_A$ receptors are the molecular substrates mediating precipitation of narcosis but not of sedation by the combined use of diazepam and alcohol in vivo. Eur J Neurosci 18:2599-2604.

Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11:823-836.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ (2003) The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur J Neurosci 17:13-27.

Todd AJ, Spike RC, Polgar E (1998) A quantitative study of neurons which express neurokinin-1 or somatostatin sst2a receptor in rat spinal dorsal horn. Neuroscience 85:459-473.

Todd AJ, Sullivan AC (1990) Light microscope study of the coexistence of GABA like and glycine like immunoreactivities in the spinal cord of the rat. J Comp Neurol 296:496-505. Treede RD, Kenshalo DR, Gracely RH, Jones AKP (1999) The cortical representation of pain. Pain 79:105-111.

Tretter V, Jacob TC, Mukherjee J, Fritschy JM, Pangalos MN, Moss SJ (2008) The clustering of $GABA_A$ receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor 2 subunits to gephyrin. J Neurosci 28:1356-1365.

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K (2003) P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424:778-783.

Tucker AP, Lai C, Nadeson R, Goodchild CS (2004) Intrathecal midazolam I: a cohort study investigating safety. Anesth Analg 98:1512-1527.

van Rijnsoever C, Täuber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM, Crestani F (2004) Requirement of 5-$GABA_A$ Receptors for the Development of Tolerance to the Sedative Action of Diazepam in Mice. J Neurosci 24:6785-6790.

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE (2001) $GABA_A$ receptor alpha1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. J Neurosci 21:3009-3016.

Waldvogel HJ, Baer K, Eady E, Allen KL, Gilbert RT, Möhler H, Rees MI, Nicholson LF, Faull RL (2010) Differential localization of γ-aminobutyric acid type A and glycine receptor subunits and gephyrin in the human pons, medulla oblongata and uppermost cervical segment of the spinal cord: an immunohistochemical study. J Comp Neurol 518:305-328.

Whiteside GT, Munglani R (2001) Cell death in the superficial dorsal horn in a model of neuropathic pain. J Neurosci Res 64:168-173.

Wieland HA, Lüddens H, Seeburg PH (1992) A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Willis Jr WD (1999) Dorsal root potentials and dorsal root reflexes: a double-edged sword. Exp Brain Res 124:395-421.

Wisden W, Gundlach AL, Barnard EA, Seeburg PH, Hunt SP (1991) Distribution of $GABA_A$ receptor subunit mRNAs in rat lumbar spinal cord. Brain Res Mol Brain Res 10:179-183.

Witschi R, Johansson T, Morscher G, Scheurer L, Deschamps J, Zeilhofer HU (2010) Hoxb8-Cre mice: A tool for brain-sparing conditional gene deletion. Genesis 48:596-602.

Witschi R, Punnakkal P, Paul J, Walczak JS, Cervero F, Fritschy JM, Kuner R, Keist R, Rudolph U, Zeilhofer HU (2011) Presynaptic Alpha2-$GABA_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control. J Neurosci 31:8134-8142.

Woolf CJ (2010) What is this thing called pain? J Clin Invest 120:3742-3744.

Yasaka T, Tiong SYX, Hughes DI, Riddell JS, Todd AJ (2010) Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. Pain 151:475-488.

Yasaka T, Kato G, Furue H, Rashid MH, Sonohata M, Tamae A, Murata Y, Masuko S, Yoshimura M (2007) Cell-type-specific excitatory and inhibitory circuits involving primary afferents in the substantia gelatinosa of the rat spinal dorsal horn in vitro. J Physiol 581:603-618.

Zeilhofer HU, Zeilhofer UB (2008) Spinal dis-inhibition in inflammatory pain. Neurosci Lett 437:170-174.

Zoli M, Jansson A, Syková E, Agnati LF, Fuxe K (1999) Volume transmission in the CNS and its relevance for neuropsychopharmacology. Trends Pharmacol Sci 20:142-150.

Zylka MJ (2005) Nonpeptidergic circuits feel your pain. Neuron 47:771-772.

6 Appendices

6.1 Abbreviations

ACC	Anterior cingulate cortex
aCSF	artificial cerebrospinal fluid
BDNF	Brain derived neurotrophic factor
BDZ	Benzodiazepines
CCI	Chronic constriction injury
CGRP	Calcitonin-gene related peptide
COX-2	Cyclooxygenase-2
CVLM	Caudal ventrolateral medulla
DAB	Diaminobenzidine tetrahydrochloride
DRG	Dorsal root ganglion
DRPs	Dorsal root potentials
DZP	Diazepam
EPSC	Excitatory postsynaptic currents
ERKs	Extracellular signal-regulated kinases
$GABA_AR$	$GABA_A$ receptors
$GABA_BR$	$GABA_B$ receptors
GAD	Glutamate decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GLYT2	Glycine transporter type 2
i.p.	Intraperitonial
i.t.	Intrathecal
IB4	Isolectin B4
IR	Immunoreactivity
LPb	Lateral parabrachial area
LTP	Long term potentiation
mPGES-1	microsomal prostaglandin E synthase-1

MRGPRD	Mas-related G-protein-coupled receptor member D
NGF	Nerve growth factor
NK1R	Neurokinin 1 receptor
NKCC1	Sodium-potassium-chloride transporter
NO	Nitric oxide
NTS	Nucleus of the solitary tract
PAD	Primary afferent depolarization
PAG	Periaqueductal grey matter
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PWL	Paw withdrawal latencies
PWT	Paw withdrawal thresholds
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RVM	Rostroventromedial medulla
SNI	Spared nerve injury
sns	sensory neuron specific sodium channel
TBST	Tris buffered saline-Tween 20
TRPV-1	Transient receptor potential vanilloid- 1 receptor
TTX	Tetrodotoxin
VGCCs	Voltage-gated calcium channels
VGLUT1	Vesicular glutamate transporter 1
VIAAT	Vesicular inhibitory amino acid transporter

6.2 Curriculum Vitae

Personal information

Family/ First name	Paul/Jolly Mrs.
Date of birth	May 30, 1981
Nationality	Indian
Current Work Address	Institute of Pharmacology and Toxicology University of Zurich Winterthurerstrasse 190 8057 Zurich
E-mail	jolly.paul@pharma.uzh.ch/ jollypaul@gmail.com

Education

2008 - Present	Ph.D. student Institute of Pharmacology and Toxicology University of Zurich, Switzerland Topic: Organization and plasticity of $GABA_A$ receptor subtypes in spinal nociceptive pathways. Advisors: Prof. Jean Marc Fritschy & Prof. Hanns Ulrich Zeilhofer
2001 - 2004	Mahatma Gandhi University, India Master of Biotechnology GPA-2.9/4.0 (First Class) Thesis: The role of serotonin and adrenaline receptor subtypes in adult hippocampal neurogenesis
1998 - 2001	Mahatma Gandhi University, India Bachelor of Biological Techniques and Specimen Preparation GPA- 4.0/4.0 (First Rank), 1996 – 1998 Thesis: Entrepreneurial project charter preparation on establishing a Biological analytical laboratory.

Professional Experience

01.2007 – 04.2008	Tata Institute of Fundamental Research (TIFR), India Senior Research Fellow Duties: Project & lab manager, technician
08. 2004 – 12.2006	Central Food Technological Research Institute (CFTRI), India, Senior & Junior Research Fellow Duties: Project & lab manager, technician
01. 2004 – 06.2004	C.M.S. College, Mahatma Gandhi University, India Guest Lecturer, Topics: Biotechnology and Botany

6.3 Publications

1. Witschi R*, Punnakkal P*, **Paul J***, Walczak B, Cervero F, Fritschy JM, Kuner R, Ruth Keist, Rudolph U, Zeilhofer HU (2011) Presynaptic α2GABA$_A$ receptors in primary afferent depolarization and spinal pain control. Journal of Neuroscience. 31(22): 8134-8142. * Equal contribution.

2. Röhn TA, Ralvenius WT, **Paul J**, Borter P, Hernandez M, Witschi R, Grest P, Zeilhofer HU, Bachmann MF, Jennings GT (2011) A VLP-based anti-NGF vaccine reduces inflammatory hyperalgesia: Potential long-term therapy for chronic pain. Journal of Immunology. 186(3):1769-1780.

3. Mangale VS., Hirokawa KE.,. Satyaki PRV, Gokulchandran N, Chickbire S, Subramanian L, Shetty AS., Martynoga B, **Paul J**, Mai MV, Li Y, Flanagan LA, Tole S, Monuki ES (2008) Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. Science. 319(5861): 304 – 309.

4. **Paul J**, Prapulla SG (2007) A Process for the production of multienzyme systems by fermentation. Govt. of India patent: 656/del/07.

Manuscripts in Preparation

1. **Paul J**, Yévenes GE, Benke D, Di Lio A, Crestani F, Witschi R, Cook J, Rudolph U, Fritschy JM, Zeilhofer HU. Antihyperalgesic actions of α2 GABA$_A$ receptors occur through a spinal site of action.
2. **Paul J**, Witschi R, Zeilhofer HU, Fritschy JM. Selective distribution of GABA$_A$ receptor subtypes in mouse spinal dorsal horn neurons and primary afferents.

6.4 Poster Presentations

1. Presynaptic α2 $GABA_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control. Society for Neuroscience 2011, Washington D.C.
2. Presynaptic α2 $GABA_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control. Swiss Society of Pharmacology and Toxicology 2011, Zurich.
3. Presynaptic α2 $GABA_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control. Neuroscience Center Zurich (ZNZ) symposium 2011, Zurich.
4. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Society for Neuroscience 2010, San Diego.
5. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Federation of European Neurosciences 2010, Amsterdam.
6. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Swiss society for Neuroscience 2010, Lausanne.
7. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Neuroscience Center Zurich (ZNZ) symposium 2010, Zurich.
8. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Swiss society for Neuroscience 2009, Fribourg.
9. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Neuroscience Center Zurich (ZNZ) symposium 2009, Zurich.
10. Molecular diversity of $GABA_A$ receptors in nociceptors terminals of mouse spinal cord dorsal horn. Pharmacology poster day 2009, Zurich.
11. Molecular organization of GABA receptor subtypes in spinal cord dorsal horn in relation to chronic pain. Neuroscience Center Zurich (ZNZ) symposium 2008, Zurich.